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# Expression of Toll-like receptors in nasal epithelium in allergic rhinitis

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Toll-like receptors (TLRs) are important in barrier homeostasis, but their role in airborne allergies is not fully understood. The aim was to evaluate baseline and allergen-induced expression of TLR proteins in nasal epithelium during allergic rhinitis. Nineteen otherwise healthy non-smoking volunteers both allergic to birch pollen and non-allergic controls were enrolled. We took nasal biopsies before and after off-seasonal intranasal birch pollen or diluent challenge. The expression of epithelial TLR1-7, TLR9-10, and MyD88 proteins was immunohistochemically evaluated from the nasal biopsies. The TLR1-3 and TLR5-10 mRNAs were observed by RNA-microarray. Baseline epithelial expression of TLR proteins was wide and identical in controls and atopics. After off-seasonal intranasal birch pollen challenge, a negative change in the expression score of TLR1 and TLR6 proteins was detected in the atopic group. TLR mRNA expression was not affected by birch pollen challenge. Nasal epithelium seems to express all known TLRs. The mechanisms by which TLR1, and TLR6 proteins could affect pollen allergen transport need further studies.

Key words: Allergic rhinitis; challenge; epithelium; immunohistochemistry; toll-like receptor.

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Birch pollen allergic rhinitis is the most common allergic disorder in the Northern Europe, with a prevalence of 15–20% (1, 2). Epigenetic and genetic modifications of innate immunity together with microbial and other environmental stimuli may predispose to airway allergy (3, 4). Many intrinsic and environmental factors facilitate the entry of airborne allergens in the respiratory mucosa (3). Epithelial cells produce mediators, which affect the recruitment and activation of more specialized immune cells and create a microenvironment where these activated immune cells may function and propagate the inflammatory processes (5).

Innate immunity by pathogen recognition is a pivotal defense system. Its aim is fast detection of pathogens from the environment when they get into contact with the organisms. At least four classes of pattern recognition receptors (PRRs) identified to date are critical for sensing microorganisms and for the subsequent stimulation of proinflammatory responses: toll-like receptors (TLRs), nucleotide oligomerization domain-like receptors, RIG-I-like receptors, and C type lectin receptors (6). Human TLRs are a large family with at least eleven members (5). TLRs sense a large diversity of pathogen-associated molecular patterns from various intruders such as bacteria and viruses, bacterial lipopolysaccharide (LPS), lipoproteins, peptidogly-

cans, bacterial DNA, and double-stranded RNA (7–10). TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 are expressed on the epithelial cell surface and recognize the pathogen-associated molecular patterns of extracellular microbes (8, 11–13). TLR3, TLR7, TLR8, and TLR9 are localized within the intracellular endolysosomal compartments and are involved in the recognition of nucleic acids. TLR3 utilizes exclusively the TRIF-dependent pathway. The signals of other TLRs utilize the MyD88-dependent pathways as well (14). For instance, upon recognition of LPS on the cell surface, TLR4 first induces the TIRAP/MyD88 signaling on the plasma membrane and is thereafter endocytosed. Next TRAM-TRIF is activated in early endosomes leading to induction of type-I interferons (7–11).

Under normal conditions commensal bacteria are recognized by TLRs and this recognition is essential for the maintenance of homeostasis and a state of constant controlled inflammation (15). Different mutations and experimental models, which alter the TLR functions, have demonstrated the significance of TLRs in susceptibility to infection (16–18). TLRs are also reported to be involved in the pathogenesis of a large number of inflammatory disorders, such as asthma and allergy (13), chronic rhinosinusitis (19), inflammatory bowel disease (20), atherosclerosis (21), and obesity (22). Meta-analyses of genome-wide studies indicate that loci in the region TLR1-TLR6-TLR10 might associate with atopic sensitization or reported allergy (23, 24). Der p 2, the main house dust mite allergen, has shown to mimic MD2 like the chaperone that promotes TLR4 signaling (25). Previous studies show that the following TLRs might be related with grass or birch pollen allergic rhinitis: TLR1, TLR2, TLR6, TLR7, TLR8, and TLR10 (23, 26–29).

We previously demonstrated that birch pollen allergic patients might have reduced immune response in their nasal epithelium (30). This could putatively lead to an active epithelial transport of birch pollen allergens detected only in patients allergic to birch pollen (31). The aim of this study was to evaluate whether the baseline epithelial expression of TLRs and MyD88 differ between healthy and birch pollen allergic subjects. Moreover, we aimed at detecting allergen-induced early alterations in the expression of TLRs and MyD88.

## MATERIALS AND METHODS

### Subjects

This study was carried out at the University of Helsinki and Tampere University Hospital in 2007-08. It was approved by the Ethical Committee of the Hospital District of Pirkanmaa (nro. R04044), and was performed according to the Declaration of Helsinki. Written informed consent was obtained from all subjects. Nineteen subjects (nine allergic and 10 healthy) participated in the study (Table 1). The subjects were Caucasian and were either atopic with allergic rhino-conjunctivitis symptoms, or non-atopic. The diagnosis of birch-induced allergic rhinitis was based on a history of spring seasonal allergic rhinitis, clinical examination, and skin prick test positivity according to ARIA-guidelines (1). Characteristics of the subject groups are shown in Table 1. Exclusion criteria were smoking, acute respiratory infection during the experiment, other diseases than allergic rhinoconjunctivitis, regular use of medication, as well as nasal endoscopic findings of moderate or severe septal deviation, nasal polyps, or mucopurulent discharge.

### Nasal challenge and biopsies

The subjects participated in the challenge experiment in January, which is the season with no flowering outdoor plants in Finland. The local anesthesia and biopsy techniques have been previously described (32). Briefly, the first biopsy was taken with Fokkens′ forceps from the anterior edge of the right inferior turbinate before the challenge. The challenge was performed by putting 3–5 drops of either birch pollen solution (Betula Verrucosa, Alutard SQ, 10 000 SQ-U, ALK-Abelló, Hørsholm, Denmark) or diluent (ALK-Diluent; ALK-Abelló) on the left nasal inferior turbinate. The second biopsy was taken from the left inferior turbinate 3 min after challenge. The patients were asked not to use medication (antihistamine and/or nasal corticosteroids) for a minimum of 5 days before specimens were taken. However, all subjects reported that they had not needed to use medication for a longer time than 4 weeks before the specimens were taken.

### Symptoms scores

The patients were asked the following symptoms before and 20 min after taking the biopsies: nasal and eye itch-

**Table 1.** The antibodies (all IgG) and their concentrations used in immunohistochemistry for detecting TLR 1-7 and 9-10 proteins. The mAb anti-TLR8 was not commercially available when the work was performed, and thus was not used

TLR1 (H-90):sc-30000 Santa Cruz Biotechnology, Inc., Dallas, Texas, USA, 1:100, 2 μg/mL
TLR2 (H-175):sc-10739 Santa Cruz Biotechnology, Inc., 1:50, 4 μg/mL
TLR3 (H-125):sc-10740 Santa Cruz Biotechnology, Inc. 1:50, 4 μg/mL
TLR4 (H-80):sc-10741 Santa Cruz Biotechnology, Inc. 1:50, 4 μg/mL
TLR5 (IMG-664A) Imgenex, San Diego, California, USA, 1:200, 2.5 μg/mL
TLR6 (IMG-304A) Imgenex, 1:3000, 0.17 μg/mL
TLR7 (IMG-581A) Imgenex, 1:300, 1.67 μg/mL
TLR9 (H-100):sc-25468 Santa Cruz Biotechnology, Inc. 1:100, 2 μg/mL
TLR10 (DDX0490) Dendritics, Lyon, France, 1:200, 2.5 μg/mL
MyD88 (ab2068) Abcam, Cambridge, UK, 1:800, 1.25 μg/mL

ing; nasal congestion, discharge, sneezing, pain, and bleeding. The scores for itching symptoms and congestion-discharge-sneezing symptoms were used in this study. These two scores were determined from the asked corresponding symptoms semiquantitatively 0 (no symptoms), 1 (mild), 2 (moderate), 3 (severe).

### Immunohistochemistry and light microscope evaluation

The nasal biopsies were formalin-fixed and paraffin-embedded. Four μm sections of paraffin blocks were deparaffinized in xylene and rehydrated in decreasing concentration of ethanol to distilled water. Slides were pretreated in a PreTreatment module (Lab Vision Corp., Fremont, CA, USA) in Tris-HCl (pH 8.5) for antigen retrieval and stained in an Autostainer 480 (Lab Vision Corp.). Primary antibodies are listed in Table 1. For detection of bound antibodies Dako REAL EnVision Detection system, Peroxidase/DAB+, Rabbit/Mouse (Dako, Glostrup, Denmark) was used. Slides were counterstained with hemalaun-eosin. Mucosa of the oral and nasal cavities, placenta and pancreas served as positive controls. The specificity of immunohistochemistry was controlled by omitting the primary antibodies. Two researchers (JR and JH) scored the stained sections independently. Cases of disagreement were discussed, and a consensus score was determined for further analysis. The staining score was determined semiquantitatively from the samples: 0 (no positively stained cells); 1 (< 5–20% of the cells were positive); 2 (20–50% of the cells were positive); 3 (> 50–80% of the cells were positive); 4 (80–100% of the cells were positive). For data analyses, we used the staining scores and the delta staining scores. The delta staining score was counted in the following way: the delta staining score – staining score $_{postchallenge}$ – staining score $_{prechallenge}$. Inflammation in the nasal specimens was based on the semiquantitatively assessed amount of inflammatory cell (lymphocyte and polymorphonuclear leukocyte) infiltration in the mucosa, and was scored by: 0 = no inflammation, 1 = mild inflammation, 2 = moderate inflammation, 3 = strong inflammation.

### mRNA levels of TLR1-10

For the RNA-microarray assay, we used nasal biopsies taken from six healthy and seven allergic subjects before and 3 min after the intranasal birch or diluent challenge. For RNA isolations Rneasy Mini kit (Qiagen, Hilden, Germany) was used according to the instructions of the manufacturer. RNA integrity and quantity were measured with AgilentBioanalyser RNA 6000 Nano kit and NanoDrop spectrophotometer, respectively. The processed RNA samples were prepared and hybridized on Illumina human WG-6 v2 chips in Finnish DNA Microarray Centre (Turku Centre for Biotechnology, Turku, Finland). Chipster analysis software was used for basic statistics and data normalization (quantile) purposes and IPA software (Ingenuity; Qiagen) comparing the groups of samples and interpreting the data, respectively (33).

### Data analysis

Statistical analysis was carried out by the PASW statistics 18.0 Statistical Software Package (SPSS Inc., Chicago, IL, USA). Data are expressed as means and as medians when specified. For comparisons, the results were analyzed by, Fisher's exact test (discrete) or Kruskal–Wallis and Mann–Whitney $U$ (MWU) tests (continuous). For pairwise comparisons Wilcoxon test was used. Two-tailed p-values of < 0.05 were considered statistically significant.

## RESULTS

### The baseline nasal epithelial expression of TLR proteins in healthy and allergic subjects

The atopic and control groups did not differ by age, male-female ratio, or by number and percentage of peripheral blood eosinophils (p < 0.05, Table 2). Atopic patients had significantly increased total IgE, birch specific IgE, and timothy specific IgE levels in serum (Table 2). We detected expression of TLR 1-7 and 9-10 proteins in nasal epithelial layer and glandular epithelial cells (Figs 1 and 2). In addition, mild expression of TLR 3 and TLR5-7 was detected in mucosal leukocytes and endothelial cells. In this study we focused on the epithelial expression. In winter and before challenge, median epithelial staining scores of TLR 1-7 and TLR 9-10 proteins did not differ between the atopic and control groups (p > 0.05, by Mann–Whitney $U$-test, data not shown). The expression of TLR1 was moderate and granular, and mostly found in the lower half of the nasal epithelium. The expression of TLR2 was weak and found close to nuclei, whereas that of TLR3 was strong, granular and detected in all epithelial compartments. The expression of TLR4 and TLR5 was moderate and unevenly distributed in the epithelium. Interestingly, a strong expression of TLR6 was found throughout the whole nasal epithelium in both groups. A moderate TLR7 expression was mainly detected in the vicinity of the nucleus borders. The expression of TLR9 and TLR10 was moderate and granular throughout all nasal epithelial layers. The expression of MyD88 was patchy and varied from moderate to strong. Atopic female subjects had a lower staining score of TLR2, median (min–max) 0 (0–1); than atopic male subjects 1 (1–3) (p = 0.04, by Fisher's exact test, data not shown). There were no other gender- or age-related differences in the baseline expression of TLR proteins (p > 0.05 by Fisher's exact and Mann–Whitney $U$-tests correspondingly, data not shown).

### The nasal epithelial expression of TLR proteins and genes after challenge

After intranasal challenge with birch pollen solution, the median staining score of TLR6 was signifi-

**Table 2.** Patient characteristics. Q1 and Q3 indicate 25% and 75% percentiles, respectively. p-values by Fisher's exact test (continuous variables); or by Kruskal–Wallis and Mann–Whitney $U$-tests (dichotomous variables)

| | Control N = 10 | Atopy N = 9 | p-Value |
|---|---|---|---|
| Age | | | |
|   Median | 23 | 24 | 0.82 |
|   Min–max | 22–36 | 22–34 | |
| No. of male sex | 3 | 3 | 1.00 |
| Blood eosinophil count ($10^9$/L) | | | |
|   Median | 0.09 | 0.13 | 0.66 |
|   Q1–Q3 | 0.08–0.19 | 0.10–0.24 | |
| Serum total IgE (IU/mL) | | | |
|   Median | 27.0 | 98.0 | 0.013 |
|   Q1–Q3 | 11.5–72.0 | 57.0–694.0 | |
| S-IgE birch(IU/mL) | | | |
|   Median | <0.35 | 12.0 | 0.001 |
|   Q1–Q3 | <0.35–<0.35 | 8.0–192.5 | |
| S-IgE timothy grass(IU/mL) | | | |
|   Median | <0.35 | 5.9 | 0.002 |
|   Q1–Q3 | <0.35–<0.35 | 3.5–16.5 | |
| No. subjects with positive SPT reaction to | | | |
|   Any aeroallergen | 0 | 9 | <0.001 |
|   Birch pollen | 0 | 9 | <0.001 |
|   Timothy grass pollen | 0 | 5 | 0.008 |
|   Other grass pollen | 0 | 7 | <0.001 |
|   Animal dander | 0 | 5 | 0.008 |
|   House dust mite | 0 | 0 | 1.00 |
|   Other aeroallergens | 0 | 0 | 1.00 |
| No. patients challenged with | | | |
|   Diluent | 4 | 3 | 1.00 |
|   Birch | 6 | 6 | |
| Itching symptom score median (min–max) | | | |
|   Before challenge | 0 (0–0) | 0 (0–0) | 1.00 |
|   After diluent | 0 (0–0) | 0 (0–0) | 1.00 |
|   After birch | 0 (0–0) | 1 (0–2) | 0.001 |
| Congestion-discharge-sneezing score median (min–max) | | | |
|   Before challenge | 0 (0–0) | 0 (0–0) | 1.00 |
|   After diluent | 0 (0–1) | 1 (0–1) | 0.49 |
|   After birch | 0 (0–1) | 0.5 (0–1) | 1.00 |
| Mucosal inflammation score median (min–max) | | | |
|   Before challenge | 1 (0–2) | 1 (1–2) | 1.00 |
|   After diluent | 2 (1–2) | 2 (1–2) | 1.00 |
|   After birch | 1 (0–2) | 1.5 (1–2) | 0.55 |

cantly lower in the atopic group contrasted to the healthy group (p = 0.02, Fig. 3), whereas no difference was observed after the diluent challenge. No differences were observed in the expression of TLR1-5, TLR7, TLR9-10, and MyD88 between the groups. There was no change in the median staining score of TLR1-7 and 9-10 proteins between nasal biopsies taken before and 3 min after the intranasal challenge in winter (p > 0.05, by Wilcoxon test, data not shown). The finding was similar in control and allergic groups, and after challenge with either birch pollen or diluent drops. The median delta staining scores of TLR1 and TLR6 were significantly lower in the atopic compared to the control group, but only after challenge with birch pollen (p = 0.04 both, Fig. 3). Interestingly, after

the diluent challenge, the median delta staining score of TLR5 in the atopic group was 0 indicating no change, whereas in the control group it was 1.5 reflecting an increased TLR5 expression (p = 0.03 Fig. 3). The median delta staining scores of TLR2-5, TLR7, TLR9-10, and MyD88 did not differ between control and atopic groups, after challenge with birch pollen solution (p > 0.05, by Mann–Whitney $U$-test, data not shown). After the challenge with a diluent solution, the median delta staining scores of TLR1-4, TLR6-7, TLR9-10, and MyD88 did not differ between the control and atopic groups (p > 0.05, by Mann–Whitney $U$-test, data not shown). When comparing nasal biopsies taken from the same individuals before and after the challenge with either diluent or birch pollen
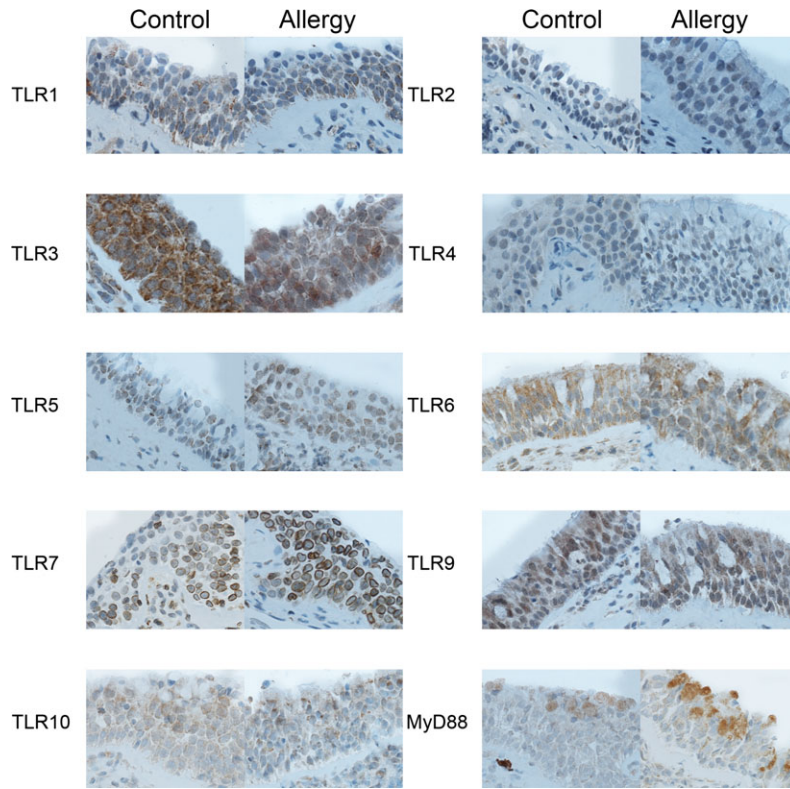
**Fig. 1.** The baseline expression of the TLR1-7 and TLR9-10 proteins in the nasal epithelium from healthy controls (Control) and subjects with birch pollen allergic rhinitis (Allergy) during winter. Magnification ×100 in all panels, except in TLR2 A, TLR 3 Control and Allergy; and TLR6 Allergy, where magnification is ×200.



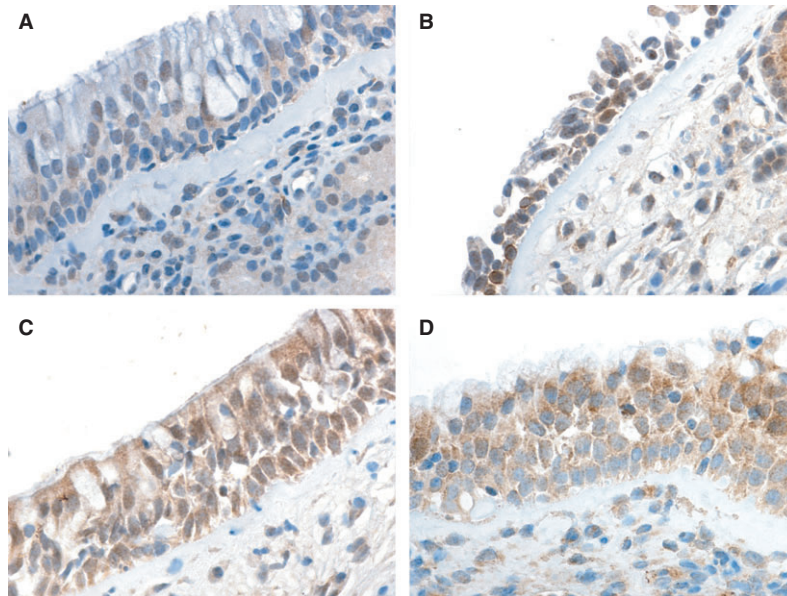**Fig. 2.** The intra-epithelial expression of TLRs. The expression of TLR2 (A) is weak close to nuclei. The expression of TLR4 (B) is moderate and unevenly distributed in the epithelium. The expression of TLR9 (C) and TLR10 (D) is granular throughout the epithelium. Magnification is ×600 in all panels.
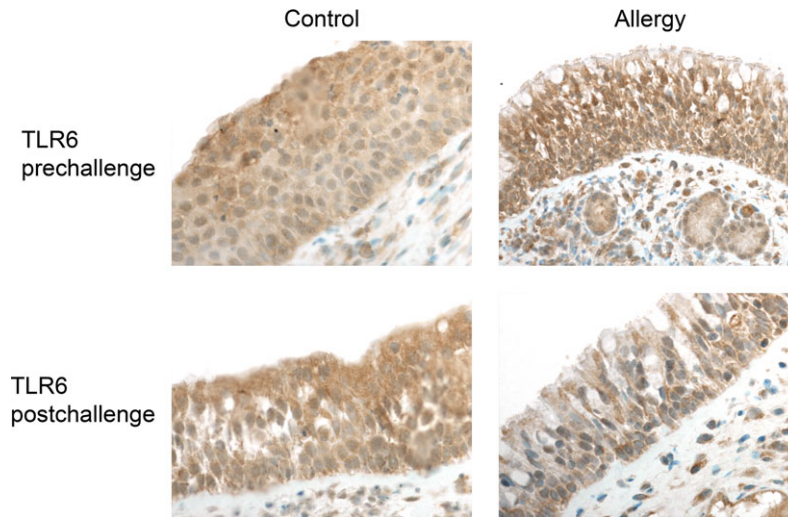
**Fig. 3.** The expression of TLR6 in the nasal epithelium from a healthy subject (Control) and a birch pollen allergic (Allergy) subject in winter. The expression of TLR6 decreased significantly in the allergic group after the birch pollen challenge. Prechallenge – the nasal biopsy was taken before intranasal challenge with the birch pollen solution. Postchallenge – the nasal biopsy was taken 3 min after intranasal challenge with the birch pollen solution. Magnification ×200 in all panels.

allergen drops the median staining scores of TLR 1-7 and 9–10 proteins, and MyD88 did not differ ($p > 0.05$, by Wilcoxon test, data not shown).

The fold change values of TLR 1-10 mRNAs ranged between 0.89 and 1.07, which indicates very slight changes on mRNA levels after challenge (Fig. 4). The median fold change of TLR8 mRNA was statistically significantly higher in allergic than control subjects after challenge with birch pollen, however the changes in TLR8 mRNA levels remained very slight and thus the difference was not biologically significant (Fig. 4H).

**Symptom scores and inflammation scores**

In winter, the baseline median score for itching symptoms and for congestion-discharge-sneezing symptoms was identical in the control and atopic groups ($p > 0.05$ both, Table 2). Accordingly, the off-seasonal baseline inflammation score of the biopsies was identical in both groups ($p > 0.05$ in both, Table 2). There were no change in the median inflammation scores when comparing specimens taken from the same individual before and 3 min after challenge with either diluent or birch, neither in controls nor in atopics ($p > 0.05$ all, by Wilcoxon test, data not shown). The median score for itching symptoms asked 20 min after birch challenge, was significantly higher in the atopic than in the control group, whereas no difference was detected after diluent challenge ($p < 0.001$, $p = 1.00$

correspondingly, Table 2). In contrast, all subjects reported postchallenge nasal pain/irritation, discharge, or slight bleeding, probably due to biopsy-taking. Thus, there were no postchallenge differences between the atopic and control group in median score for congestion-discharge-sneezing symptoms ($p > 0.05$, Table 2). None of the subjects reported dyspnea after challenge.

**DISCUSSION**

In this study we showed that TLR 1-7 and 9-10 proteins are expressed in nasal epithelium. The TLR3, 6, 7, 10 proteins had the strongest nasal epithelial expression, whereas TLR2 had the mildest. TLR1, 2, 4, 5, 6, 10 proteins have previously been reported to be located in the plasma membrane; however we also detected them in the intracellular compartments of epithelial cells (8). Tengroth et al. demonstrated by RTq-PCR, immunohistochemistry and flow cytometry that nasal apical epithelium expresses abundantly TLR3, TLR7, TLR9, RIG-I, and MDA-5. Moreover, they showed by ELISA upregulated cytokines (IL-6, GMCSF, IL-8, IFN-b) in the nasal mucosa after stimulation of several TLR-agonists, which suggests that epithelial TLR and RLR receptors might mediate nasal viral response and thus could be important in exacerbations (34). Bielinska et al. administered intranasally anthrax antigen with a nanoemulsion to mice. This
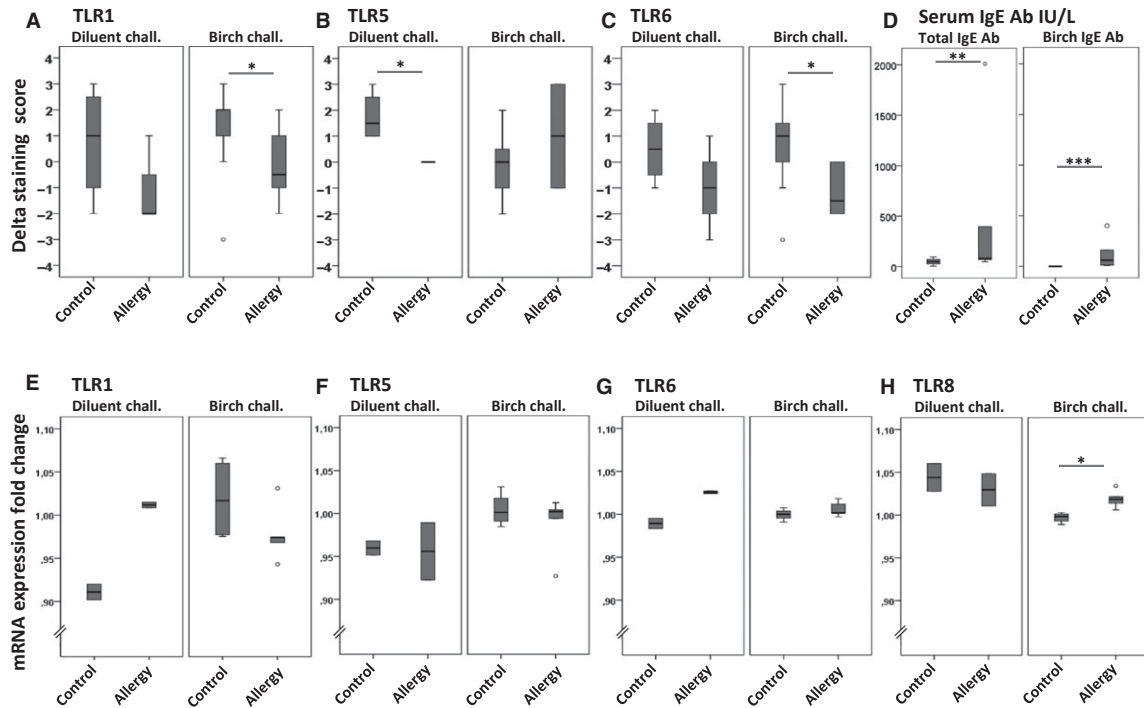
**Fig. 4.** The immunohistochemical delta staining scores of TLR proteins (A–C) and TLR mRNA fold changes (E–H). Diluent chall. – intranasal challenge with diluent solution; Birch chall. – intranasal challenge with birch pollen solution. The staining score – proportion of positively stained cells/specimen (from 0 to 4). The delta staining score = staining score $_{postchallenge}$ – staining score $_{prechallenge}$. Only the TLR proteins with statistically significantly different delta staining scores between control (Control) and birch pollen allergic (Allergy) subjects are shown: TLR1 (A), TLR5 (B), and TLR6 (C). The mRNA fold change = relative mRNA abundancy $_{postchallenge}$: relative mRNA abundancy $_{prechallenge}$. The fold changes of TLR1 (E), TLR5 (F), TLR6 (G), and TLR8 (H) mRNAs. The levels of total and birch specific IgE Ab of serum. (D) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, by Mann–Whitney $U$-test.

induced TLR2 and TLR4 activation along with a MyD88-independent antibody response and a MyD88-dependent Th-1 and Th-17 cell-mediated immune response (35). The finding might be used in the development of mucosal vaccines. Ioannidis et al. (36) demonstrated that TLR6 has a basolateral location in human tracheal epithelium which is in accordance to our observation in the nasal epithelium. TLR2-6 proteins have been demonstrated to have the strongest expression in the airway epithelium while the expression of TLR7-10 fluctuates depending on the cell type studied (5).

We previously demonstrated that birch pollen is actively transported through the epithelium within 1 min (31). In this study, we detected early changes in the expression of epithelial TLR1 or TLR6 after the birch pollen allergen challenge suggesting, that the challenge could putatively modify the structure of epithelial TLR1 or TLR6 proteins in atopic subjects leading to decreased binding of anti-TLR1 and anti-TLR6 antibodies to their epitopes. Thus, birch pollen allergen entry would putatively be regulated by TLR1 and TLR6 medi-

ated pathways. The finding that mRNA expression was not affected after the challenge, could in part be explained by the fact that whole biopsies were used instead of epithelial cells. Our future aim would be to observe if birch exposure affected the activity of TLR proteins; or noncoding RNAs regulating TLR protein synthesis.

Fransson et al. demonstrated mRNA and protein expression of TLRs 2, 3, and 4 in the nasal mucosa both in subjects with and without birch and/or timothy pollen allergy, which is in accordance to our results (29). Probably partly due to differences in the study set up, they demonstrated a more apical location of TLR3 and an increase in expression of TLRs 2, 3, 4 proteins following the allergen challenge pre-season, but an increase only in TLR3 mRNA during the pollen season (29).

Tengroth et al. have analyzed by flow cytometry and Luminex naïve nasal polyp and turbinate tissues, as well as human tissues after *in vivo* and *in vitro* stimulation with a TLR9 agonist, CpG. Interestingly, epithelial expression of TLR9 was detected in turbinates from healthy controls and

in polyp tissue, whereas TLR9 was absent in turbinates from CRSwNP patients. CpG stimulation resulted in an upregulation of TLR9 and modulation of cytokines in turbinate tissue from patients, suggesting that defects in the TLR9 mediated microbial defense in the turbinate might explain virus-induced polyp growth (37).

Several studies have observed TLRs in the atopic lower airway inflammation. TLR2-TLR7 proteins have been detected in patients with severe asthma (38). Subcutaneous QbG10, a TLR9 agonist, improved symptoms and lung function in patients with allergic asthma (39). Another placebo-controlled trial showed that repeated intranasal TLR7 agonist AZD8848 reduced symptoms in patients with birch and/or grass pollen allergic rhinitis, but it produced reversible blood lymphocyte reduction and dose-dependent flu-like symptoms as side-effects (28). Intranasal TLR9 agonist CpG increased, whereas TLR7 agonist, R848, decreased airway inflammation in mice with established allergic inflammation (40). Parsons et al. (41) showed that primary bronchial epithelial cells from asthma patients were able to up-regulate TLR3, following infection, but failed to initiate an effective innate immune response. Deifl et al. stimulated *in vitro* monocytes and monocyte-derived dendritic cells from allergic patients with TLR ligands. They found that TLR ligands except flagellin enhanced Bet v 1 –allergen uptake (42). Our finding that the diluent challenge in controls induced expression of TLR5, which usually recognizes flagellin, requires further evidence to be explained.

In mice, allergic rhinitis response to house dust mite might result from TLR2 signaling axis in the nasal mucosa, whereas in the lung mucosa the allergic asthma response occurs predominantly via TLR4 signaling axis (43). Intranasally administered TLR3 or TLR4 ligands induced relatively similar murine airway hyper-responsiveness and cellular infiltration in lungs (44). TLR2 agonist, Pam3Cys, induced an asthma exacerbation in mice, but had, on the other hand, long-term protective effect on secondary allergic responses in the airways (45).

The TLR-family with eleven members explodes in four-figure numbers when observing single nucleotide polymorphism, expression variants, and post-translational modification (46). Two meta-analyses of genome-wide association studies have shown that loci in TLR1-TLR6-TLR10 region associate with allergic sensitization; or self-reported cat, dust mite, or pollen allergies (23, 24). Single nucleotide polymorphism in the TLR1, 6, 7, 8 genes might associate with grass and/or birch pollen allergic rhinitis; of which the association between TLR7-8 gene variants and grass pol-

len allergic rhinitis was more pronounced (26, 27). Other studies have shown that polymorphisms or defects in TLR1-2, TLR6-7, TLR9-10 genes, and MyD-88 dependent pathways seem to associate with atopic asthma (13, 47–49). Our findings of epithelial TLR1-2, TLR6, TLR9, and MyD88 having putatively a role in birch pollen allergic responses is consistent with these observations. Future evidence is still needed of gene-environmental interactions affecting the TLR pathways in airway allergy.

Our results should be interpreted with caution because of the small number of patients studied. The usage of the whole biopsies and short challenge time, might have in part affected the results. We also acknowledge the fact that the effect of multiple testing could limit the significance of the results.

## CONCLUSIONS

The TLR protein family is widely located in the human nasal epithelium, which most probably reflects to the active innate immunity functions of nasal epithelium. Findings of altered TLR1 and TLR6 protein expression after birch pollen challenge still require to be validated on the protein activity level.

## CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

## AUTHORS' CONTRIBUTIONS

Sanna Toppila-Salmi and Mikko Lehtonen recruited the volunteers and performed the sampling. Jutta Renkonen, Jaana Hagström, Caj Haglund, and ST-S were responsible for immunohistochemistry and microscopy. Sakari Joenväärä, Pirkko Mattila and Ville Parviainen performed the laboratory experiments and analyses. Risto Renkonen, JR and ST-S provided the study plan, data analyses and wrote the manuscript. All authors reviewed critically the manuscript.

## ABBREVIATIONS

AR: allergic rhinitis; LPS: lipopolysaccharide; MyD88: myeloid differentiation primary response 88; PRR: pattern recognition receptor; RIG-I: retinoic acid-inducible gene I; Th-1: T-helper cell 1;

Th-2: T-helper cell 2; TIR: Toll-interleukin 1 receptor; TIRAP: toll-interleukin 1 receptor (TIR) domain containing adaptor protein; TLR: Toll-like receptor; TRAM: toll-like receptor 4 adaptor protein; TRIF: TIR-domain-containing adapter-inducing interferon-β.

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