

NOD-like receptors in the human upper airways: a potential role in nasal polyposis

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Abstract

Background: Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are newly discovered cytosolic receptors belonging to the pattern-recognition receptor family. They detect various pathogen-associated molecular patterns, triggering an immune response. The knowledge about these receptors, and their role in health and disease, is limited. The aim of the present study was to characterize the expression of NOD1, NOD2, and NALP3 in the human upper airways.

Methods: Surgical samples were obtained from patients with tonsillar disease ($n = 151$), hypertrophic adenoids ($n = 9$), and nasal polyposis ($n = 24$). Nasal biopsies were obtained from healthy volunteers ($n = 10$). The expression of NOD1, NOD2, and NALP3 was analyzed using real-time PCR and immunohistochemistry.

Results: Expression of NOD1, NOD2, and NALP3 mRNA and protein were seen in all tissue specimens. The NLR mRNA was found to be higher in nasal polyps than in normal nasal mucosa, and local steroid treatment reduced the NLR expression in polyps. In contrast, tonsillar infection with *Streptococcus pyogenes* or *Haemophilus influenzae* did not affect the NLR expression.

Conclusions: The present study demonstrates the presence of NLRs in several upper airway tissues and highlights a potential role of NLRs in chronic rhinosinusitis with polyps.

The nasal mucosa constitutes the first line of defense against foreign airborne and ingested antigens (1, 2). The nasal-associated lymphoid tissue, with the nasopharyngeal tonsil (adenoid) and the palatine tonsils as major sites of interaction, constitutes a second line of protection (3, 4). Pattern-recognition receptors (PRRs) on the mucosa and in the lymphoid tissues have the ability to detect and bind conserved microbial components initiating an immune response. The most well-known members of the PRR family are the toll-like receptors (5). They have been demonstrated in various tissues of the airways, including the nasal adenoids and palatine tonsils. They have also been found in various types of immunocompetent cells (3, 5–10). Recently, the PRR family has grown by the discovery of another set of receptors; the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). Twenty-three NLR members have been identified. Based on their N-terminal domain, they are divided into three subfamilies; caspase-activating and recruitment domain-containing NODs, pyrine domain (PYD)-containing

NACHT-, LRR-, and PYD-containing proteins (NALPs), and baculovirus inhibitor repeat (BIR)-containing neuronal apoptosis inhibitor proteins (11). The best characterized proteins are NOD1, NOD2, and NALP3. NOD1 and NOD2 recognize the bacterial peptidoglycan-derived molecules γ -D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively. Both receptors trigger an immune response via activation of NF- κ B. NALP3 responds to microbial products and host-derived danger signals by activating the caspase-1-dependent inflammasome (12–14). A role for the NALP3 inflammasome in the recognition of aluminum adjuvants (alum) has been suggested, but it is not fully established whether this interaction is NALP3-specific (15, 16). Ligands for remaining NLRs have not yet been identified. Therefore, the physiological function of these receptors remains to be explored.

We have recently demonstrated the presence of NOD1, NOD2, and NALP3 in nasal biopsies and in primary nasal epithelial cells (17). Expression has also been shown in lung

tissue (18–20). Apart from this, little is known about NLRs in human airways. The present study was designed to characterize the expression of NOD1, NOD2, and NALP3 in human palatine tonsils, adenoids, nasal polyps, and nasal biopsies. How the NLR manifestation in polyps and tonsils was affected by local steroid treatment and chronic infection, respectively, was also evaluated.

Methods

Patients and sample collection

The study was approved by the local Ethics Committee, and all subjects gave their written informed consent. Three specific tissues were investigated in this study: tonsils, adenoids, and nasal mucosa (Table 1).

Palatine tonsils were obtained from 151 patients undergoing tonsillectomy under general anesthesia. After surgery, swabs were taken for tonsillar core culture to determine the microbial flora in the tonsillar crypts. The patients were divided into three groups based on their clinical diagnosis and the outcome of the core culture. The control group consisted of 52 patients who underwent tonsillectomy because of idiopathic tonsillar hypertrophy (with substantially enlarged tonsils, no history of recurrent tonsillitis, negative cultivation test [except the normal oral flora]). The infected group consisted of patients referred to tonsillectomy because of multiple episodes (≥ 4 episodes during the year preceding surgery) of tonsillitis (small to substantially enlarged tonsils, history of recurrent tonsillitis, positive cultivation test) and was further divided based on the core culture into a group consisting of 50 patients infected with group A β -hemolytic streptococci (GAS) and 49 patients infected with *Haemophilus influenzae* (HI). Adenoids were collected from nine children undergoing adenoidectomy under general anesthesia because of enlarged adenoids causing nasal obstruction.

In the nasal mucosa group, nasal biopsies were taken from the inferior turbinate of 10 healthy volunteers as previously described (17). Nasal polyps were acquired from 24 patients diagnosed with chronic rhinosinusitis (CRS) with nasal polyps (CRS_{NP}) based on clinical symptoms (nasal

obstruction, anosmia, sneezing, rhinorrhea, itching) and endoscopic visualization of polyps (21). Polyps were divided into two groups; before and after steroid treatment. In patients supplying polyps before treatment, all steroids (systemic, inhaled, and intranasal) were withheld ≥ 6 weeks before removal. Patients providing polyps after treatment were medicated with fluticasone (200 μ g twice a day) for at least 4 weeks before surgery. Samples were taken using topical application of local anesthesia containing lidocaine hydrochloride : nafazoline (34 : 0.17 mg/ml) for 20 min.

RNA extraction and real-time RT-PCR

Tissues were homogenized in RLT buffer (Qiagen, Hilden, Germany) with 1% 2-mercaptoethanol. Total RNA was extracted using RNeasy Mini kit (Qiagen), and the quantity and quality of the RNA were measured by spectrophotometry using the wavelength absorption ratio (260/280 nm). Reverse transcription of total RNA into cDNA was performed using the Omniscript™ reverse transcriptase kit (Qiagen) with oligo(dT)16 (DNA Technology A/S, Aarhus, Denmark) in a Mastercycler personal PCR machine (Eppendorf AG, Hamburg, Germany). The reaction was carried out at 37°C for 1 h in a final volume of 20 μ l.

For tonsils, adenoids, and nasal polyps, real-time PCR was performed using Stratagene Brilliant® QPCR Mastermix (Agilent Technologies, Santa Clara, CA, USA) and FAM labeled probes for NOD1 (assay ID Hs01036721_m1), NOD2 (Hs00223394_m1), NALP3 (Hs00366465_m1), and β -actin (Hs99999903_m1) from Applied Biosystems (Foster City, CA, USA). The thermal cycler was set to perform an initial set-up (95°C for 10 min) and 45 cycles of denaturation (95°C for 15 s) followed by annealing/extension (60°C for 1 min) using Stratagene Mx3000P (Agilent Technologies). For nasal biopsies, the PCR reactions were performed as previously described (17), with the following intron-overspanning primers for detection of NOD1, NALP3, and β -actin: NOD1 fwd 5'-gtggacaactgtgtaagaatgac-3' and rev 5'-ctgtac-caggtccagaattttgc-3'; NALP3 fwd 5'-gcgatcaacaggagagaccttta-3' and rev 5'-gctgtcttctggcatatcaca-3'; β -actin fwd 5'-gccaac-cgcgagaagatg-3' and rev 5'-acggccagaggcgtacag-3'. The reactions were carried out in 95°C for 15 min, followed by 46 cycles at 94°C for 30 s and 55°C for 60 s (initially 66°C, followed by a 2°C decrease the first six cycles). For detection of Nod2 and β -actin, probes from Applied Biosystems were used as described earlier. PCR analyses for nasal biopsies were carried out on a Smart Cycler (Cepheid, Sunnyvale, CA, USA). Regardless of the method used for real-time PCR analysis, the mRNA expression was assessed using the comparative cycle threshold (Ct) method where the relative amounts of mRNA were determined by subtracting the Ct value of the NLR gene with the Ct value of β -actin (Δ Ct). The amount of mRNA is expressed as $2^{-\Delta Ct} \times 10^5$ (8, 17).

Immunohistochemistry

The morphological localization of NLR proteins in upper airway tissues was assessed using immunohistochemistry.

Table 1 Demographic data of the patients

	Number of subjects	Sex	Age, median (range)
Tonsils	151	77 M, 74 F	7 (2–44)
Control group	52	19 M, 33 F	14.5 (2–44)
GAS group	50	26 M, 24 F	7 (2–30)
HI group	49	32 M, 17 F	5 (2–35)
Adenoids	9	6 M, 3 F	4 (2–10)
Nasal polyps	24	22 M, 2 F	55 (24–82)
Control group	12	12 M	58 (32–82)
Steroid treated	12	10 M, 2 F	52 (24–71)
Nasal mucosa	10	6 M, 4 F	27 (16–45)

GAS, group A β -hemolytic streptococci; HI, *Haemophilus influenzae*; M, males; F, females.

Tonsils, adenoids, nasal polyps, and nasal biopsies were embedded in paraffin, cut in 5- μ m thick sections, mounted on glass slides, and stored at -80°C until use. The immunohistochemical staining was performed according to the Envision⁺ System-horseradish peroxidase (HRP) kit (Dako, Copenhagen, Denmark) as previously described in detail (22, 23). Briefly, the sections were incubated overnight in 4°C with a polyclonal rabbit anti-human antibody against NOD1 and monoclonal mouse anti-human antibodies against NOD2 (clone 2D9) and NALP3 (nalpy3-b), all diluted 1:20 (Abcam, Cambridge, UK). Thereafter, the sections were incubated with HRP-labeled goat anti-rabbit or goat anti-mouse polymer for 30 min, followed by 3,3-diaminobenzidine substrate-chromogen for 5 min. Finally, the slides were mounted in Faramount Aqueous Mounting Medium (Dako). As negative controls, N-series universal negative control reagents against mouse and rabbit (both from Dako) or antibody diluent were utilized. Tris-buffered saline (pH 7.6) supplemented with 0.05% Tween 20 was used for all washing steps.

Statistics

Statistical analyses were performed using GRAPHPAD PRISM 5 (San Diego, CA, USA). All data are presented as median and range, and n equals the number of independent donors. Nonparametric data were analyzed for statistical differences using Mann-Whitney t -test and for correlations using Spearman's test. A P -value of <0.05 was considered statistically significant.

Results

The presence of NOD1, NOD2, and NALP3 transcripts in human tonsils, adenoids, nasal polyps, and healthy nasal

mucosa was assessed by real-time PCR, and all results were normalized to the house-keeping gene β -actin. The expression was most prominent in nasal polyps, but moderate levels were also found in tonsils and adenoids. Even though another method for determining the mRNA expression in nasal biopsies was used (17), low levels of NOD1 and NOD2 could be seen in healthy nasal mucosa, whereas NALP3 was absent (Fig. 1A–D). We also analyzed the correlation between NOD1, NOD2, and NALP3 in the different airway tissues. A strong correlation between all NLRs was found in tonsils (Fig. 1E), along with a correlated expression of NOD1 and NOD2 as well as NOD1 and NALP3 in adenoids (Fig. 1F), and NOD1 and NOD2 in polyps (Fig. 1G). Moreover, by comparing the mRNA levels in the lymphoid tissues, the expression was found to be significantly higher in tonsils than in adenoids for NOD1 (338.9 ± 106.3 vs 404.4 ± 39.4 , $P = 0.0004$) and NALP3 (263.4 ± 54.7 vs 130.6 ± 11.8 , $P = 0.003$).

To confirm and complement the mRNA data, immunohistochemical staining was performed to demonstrate the morphological localization of the receptors. A clear immunostaining of NOD1 and NOD2 was observed in the surface epithelium and in the lymphocyte-rich germinal centers and T cell zones of the tonsils, whereas NALP3 was weakly expressed (Fig. 2A–C). In adenoids, a weak to moderate staining was seen for all receptors (Fig. 2E–G). Polyps showed an intense positive staining for all NLRs in the epithelium and in stromal cells (Fig. 3I–K). In nasal biopsies, a clear immunopositivity for NOD1 and NOD2 was seen in the surface epithelium, in seromucous glands as well as in scattered intra- and subepithelial leukocytes (Fig. 2M–O). Replacement of the primary specific antibody with mouse and rabbit isotype-matched control antibodies resulted in complete loss of staining in all specimens (Fig. 2D,H,L,P).

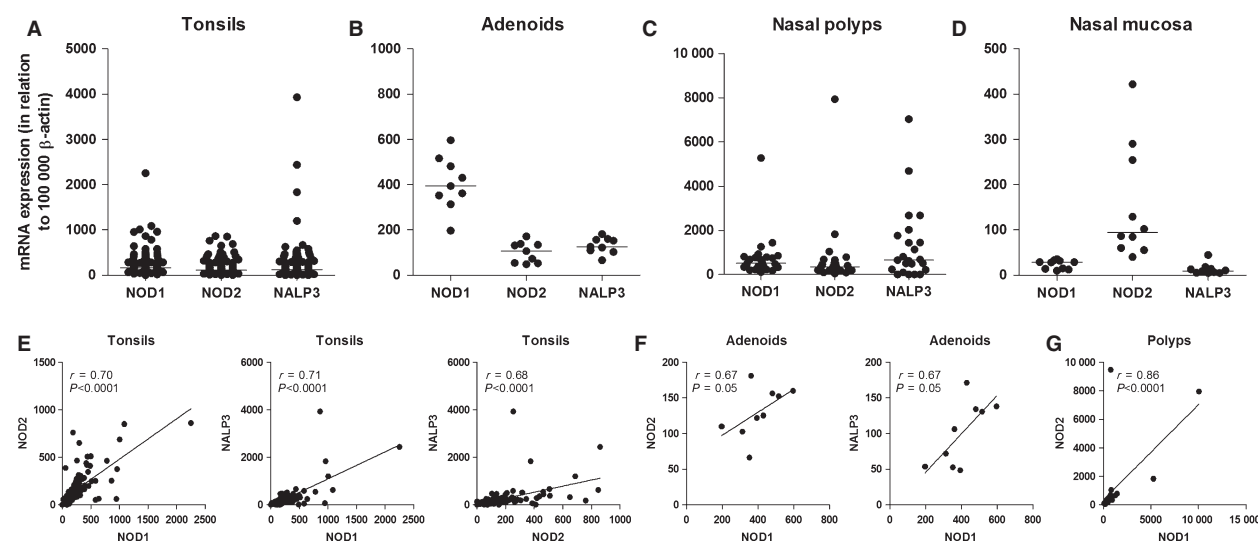


Figure 1 Expression and correlation of (NOD)-like receptors in airway tissues. Levels of NOD1, NOD2, and NALP3 in (A) tonsils ($n = 151$), (B) adenoids ($n = 9$), (C) nasal polyps ($n = 24$), and (D) healthy nasal mucosa ($n = 10$). The expression was determined by

real-time RT-PCR and depicted in relation to β -actin as $2^{-\Delta\text{Ct}} \times 10^5$. (E) The correlation between NOD1, NOD2, and NALP3 in tonsils, (F) adenoids, and (G) nasal polyps was determined using Spearman's correlation test.

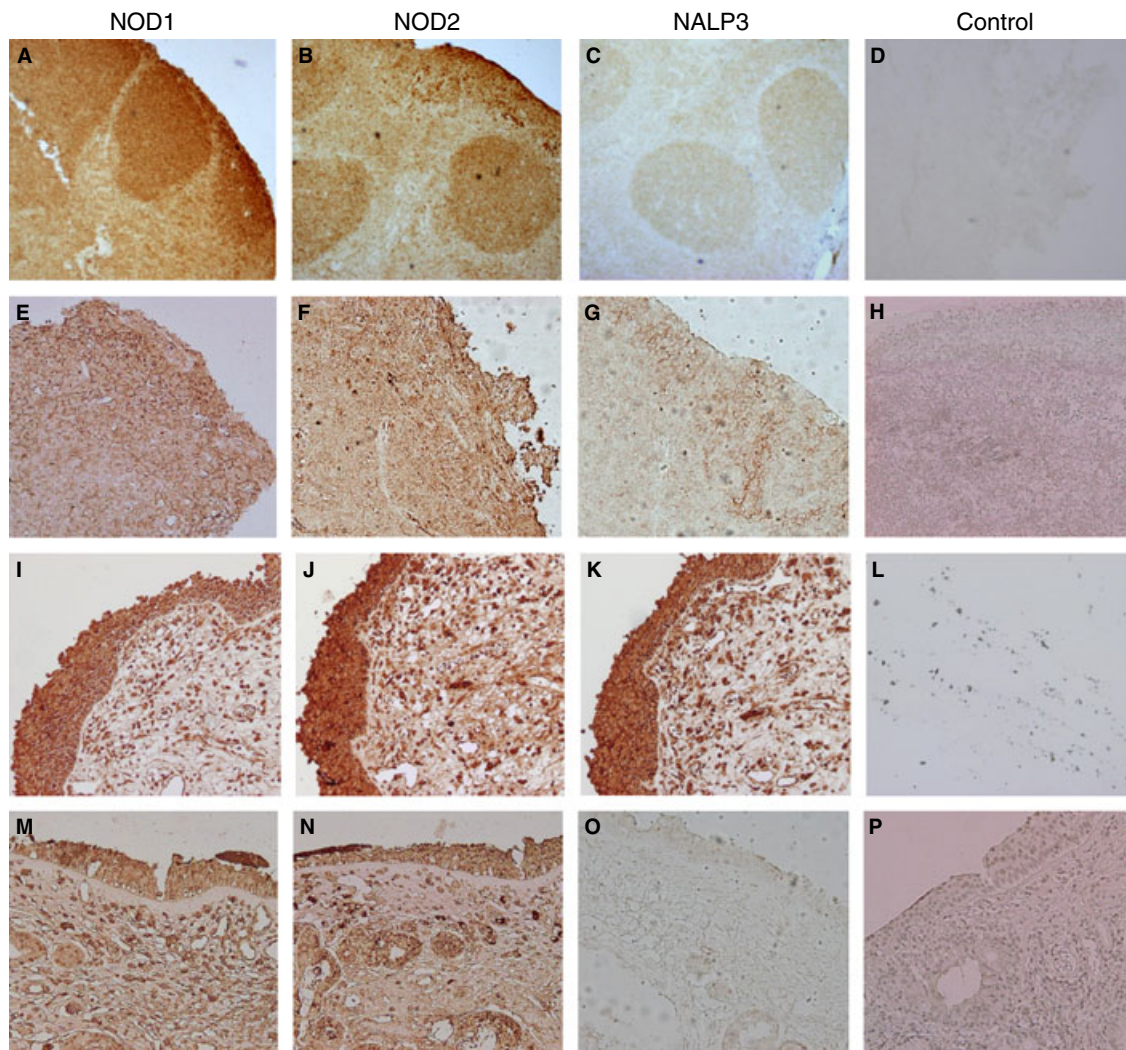


Figure 2 Immunohistochemical staining of airway tissues. Tissue slides obtained from (A–D) tonsils, (E–H) adenoids, (I–L) nasal polyps, and (M–P) nasal biopsies were incubated with antibodies against

NOD1, NOD2, and NALP3 and visualized by 3,3'-diaminobenzidine. In control slides, N-series universal negative control reagent was used. Magnification 40–100x.

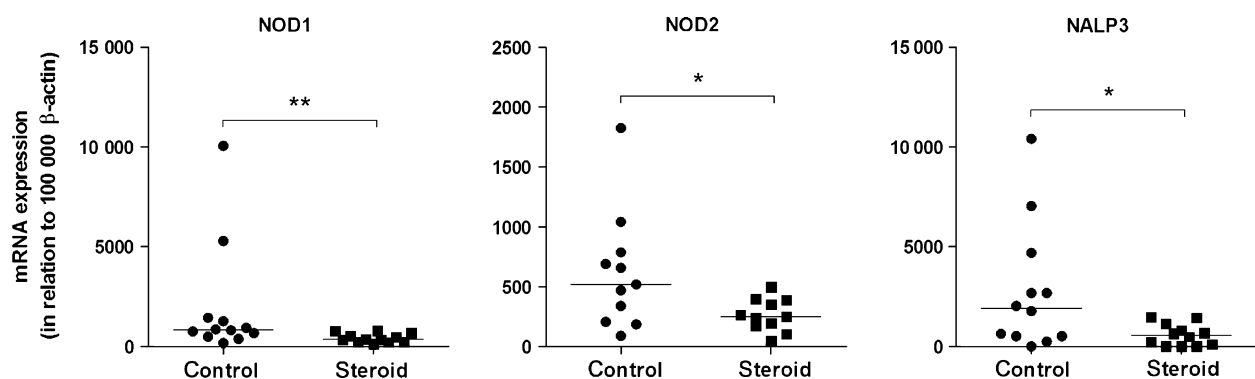


Figure 3 Topical nasal steroid treatment reduces NLR mRNA levels in polyps. The expression of NOD1, NOD2, and NALP3 was determined in nasal polyps before (control, $n = 12$) and after steroid

treatment (steroid, $n = 12$) by real-time RT-PCR. Data are presented in relation to β -actin as $2^{-\Delta C_t} \times 10^5$ and depicted as median and range. * $P < 0.05$; ** $P < 0.01$ (Mann–Whitney t -test).

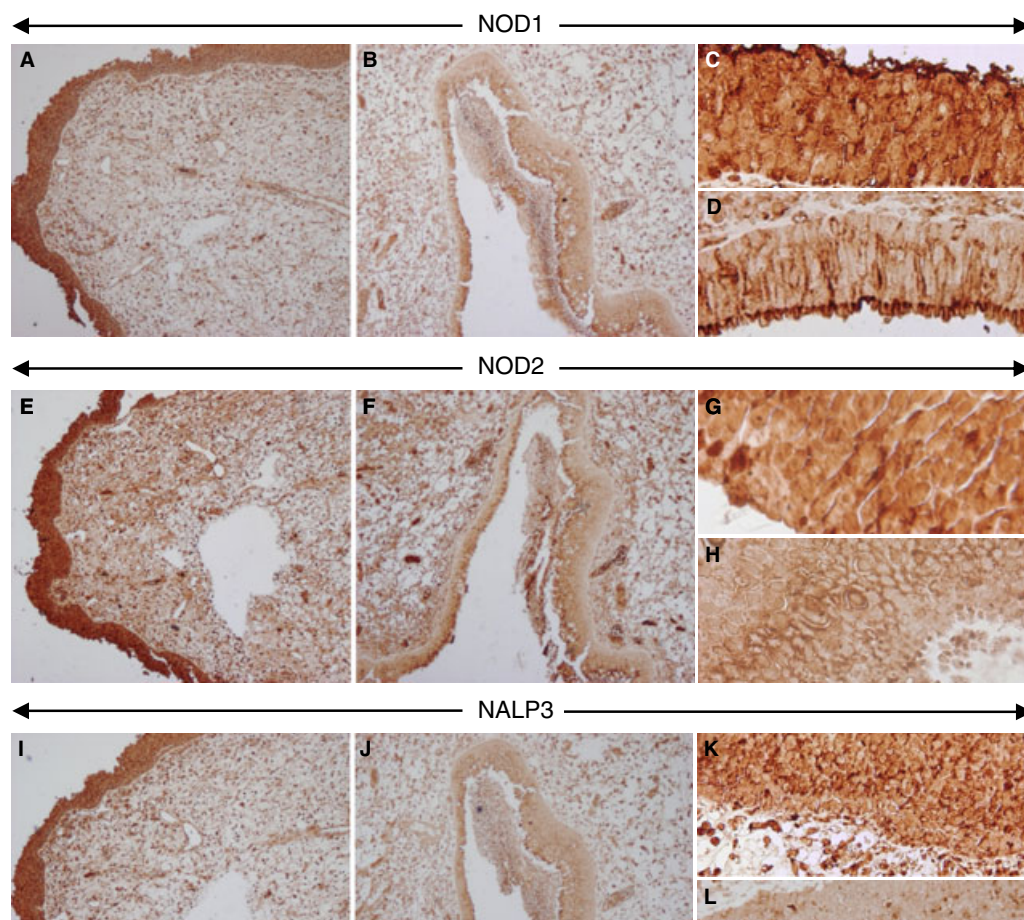


Figure 4 Reduced immunopositivity for NOD1, NOD2, and NALP3 in polyps after topical nasal steroid treatment. Nasal polyps were incubated with antibodies against NOD1, NOD2, and NALP3 and visualized by 3,3'-diaminobenzidine. (A, C) Detection of NOD1 in untreated polyps, and (B, D) polyps treated for ≥ 4 weeks with fluti-

casone. (E, G) NOD2 in untreated polyps, and (F, H) in polyps after treatment with topical steroids. (I, K) NALP3 in untreated polyps, and (J, L) steroid-treated polyps. Magnification 40 $\times$ (A, B, E, F, I, J) and 200 $\times$ (C, D, G, H, K, L).

Next, we wanted to investigate whether local steroid treatment affected the NLR expression in nasal polyps. Treatment with fluticasone for at least 4 weeks resulted in a reduction in the expression of NOD1, NOD2, and NALP3 mRNA (Fig. 3). Immunohistochemical staining of polyps before and after steroid treatment with antibodies against NLRs further confirmed this observation (Fig. 4).

Similarly, the effect of tonsillar infection on the NLR expression was assessed by comparing hyperplastic/control tonsils with tonsils infected with GAS and HI. However, no differences between the groups could be found (Fig. 5).

Discussion

The present study demonstrates mRNA and protein expression of NOD1, NOD2, and NALP3 in tonsils, adenoids, and nasal mucosa. The immunohistochemical staining revealed that these receptors primarily are found in tonsillar and

adenoid lymphocytes, in the epithelium and in conjunction with seromucous nasal glands. The mRNA levels in nasal polyps were higher than the corresponding levels found in healthy nasal mucosa. Unfortunately, the use of different methods to determine the NLR levels precluded statistical comparisons between the two groups. However, immunohistochemistry supported this finding by showing a more intense staining in nasal polyps compared with healthy nasal tissue. Four weeks of treatment with local nasal steroids induced a significant reduction in NLR levels in polyps. In contrast, multiple episodes of tonsillitis and a core culture revealing a persistent infection with GAS or HI did not seem to affect the NLR expression. Moreover, statistical analysis showed a strong correlation between the NOD1 and NOD2 expression in polyps, between NOD1 and NOD2 as well as NOD1 and NALP3 in adenoids, and a correlation between all NLRs in tonsillar tissue, suggesting parallel regulation mechanisms of the receptors.

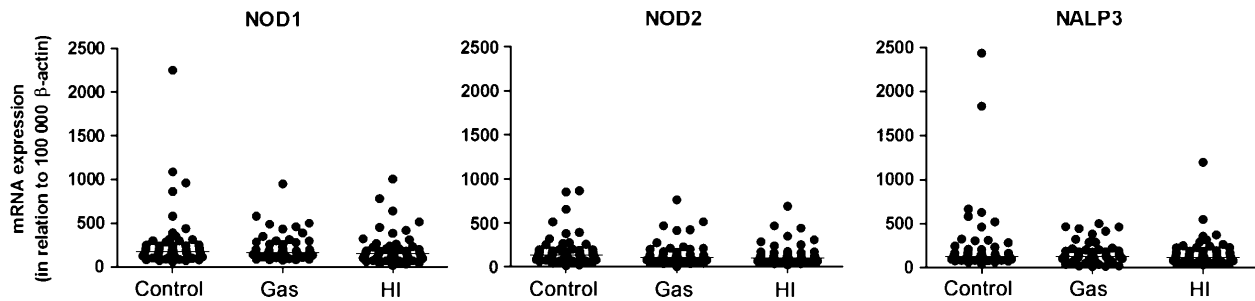


Figure 5 Tonsillar infection does not alter the NLR mRNA expression. The expression of NOD1, NOD2, and NALP3 was determined in noninfected/hyperplastic tonsils (control, $n = 52$), tonsils infected

with group A β -hemolytic streptococci (GAS, $n = 50$) and tonsils infected with *Haemophilus influenzae* (HI, $n = 49$) by real-time RT-PCR. Data are presented in relation to β -actin as $2^{-\Delta Ct} \times 10^5$.

We have previously demonstrated expression of NOD1, NOD2, and NALP3 in nasal biopsies from healthy and allergic donors and in primary nasal epithelial cells (17). In addition, we have shown expression of NOD1, NOD2, and NALP3 in human neutrophils, the most common leukocyte subset in nasal lavage fluid (24), and revealed that the NOD2 and NALP3 agonists MDP and alum, respectively, induce neutrophil activation (25). We have also demonstrated the presence of a range of NLRs in human tonsillar B cells, and that the NOD1 ligand iE-DAP is capable of inducing B cell activation upon concomitant B cell receptor triggering (26). A recent study by Lech et al. (18) showed the presence of NOD1, NOD2, and NALP3 in human lung tissue, and likewise, Swaan et al. have found NOD1 and NOD2 in human lung epithelial cells. The latter study also revealed that the NOD1 ligand iE-DAP has the ability to induce cytokine secretion (19). Another group has demonstrated expression of NOD1 and NOD2 in a range of different airway epithelial cell lines and that stimulation with the corresponding agonists induce production of β -defensin 2 (20).

A higher expression of NOD1 and NALP3 was found in tonsils compared to adenoids. A similar finding was reported by Claey's et al. (6), demonstrating a higher level of TLR2 in tonsils compared to adenoids. Moreover, as tonsils are sites of a high antigenic burden by being continuously exposed to bacteria and viruses, we wanted to investigate whether the NLR expression was correlated to the infectious state of the tonsil. Infection-induced regulations of TLR expression have been reported previously, in which microbes and/or microbial components have been found to both positively and negatively regulate the expression in various tissues and cell types (22, 27–29). However, by comparing the NLR expression in hyperplastic/control tonsils with tonsils infected with GAS and HI, no differences were seen between the groups. In support of this, the disease state of the tonsils has been reported not to affect the expression of TLR2 and TLR4 (6).

The relatively high NLR levels found in untreated nasal polyps might be explained by the fact that polyposis represents a chronic type of inflammation (30), characterized by a Th2-dominated cytokine pattern and formation of IgE (31),

during which the cells might be armed with high numbers of NLRs to respond to endogenous danger signals caused by the inflammation *per se*. Application of steroids dampens this inflammatory response, causing a down-regulation in NLR expression. This might be because of a feedback-loop that regulates both the magnitude of the inflammatory response and the expression of the NLR genes. If an inflammation-induced up-regulation of NLRs of this kind occurs in polyps, one would expect a similar up-regulation in infected tonsils. However, it should be noted that control tonsils, unlike healthy nasal mucosa, are not fully healthy tissues. Even though they are culture-negative for bacterial infection, there might be some immunological relevant cause of the hyperplasia that can affect the NLR expression in those samples. Another explanation for the differential expression might be related to differences in the anatomical and cellular composition. Unlike healthy nasal mucosa, polyps display a profound infiltration of inflammatory cells such as eosinophils or neutrophils (30, 31).

Taken together, the present study complements previous studies on PRRs in the human airways by demonstrating the presence of NLRs in tonsils, adenoids as well as in the nose, predominantly in the mucosal epithelium. Positioned at the interface between inside vs outside, the epithelium recognizes danger through its broad spectrum of PRRs (32), with the resultant release of antimicrobial peptides such as defensins, lysozyme, and S100 proteins (33). In addition, it triggers adaptive immune responses by the induction of cytokines, chemokines, and costimulatory molecules on dendritic cells and macrophages (34). This highlights the importance of the epithelium in maintaining upper airway homeostasis and indicates that failure of these defense mechanisms might lead to upper respiratory tract infections and/or allergy. Moreover, it is currently not understood whether recurrent episodes of local infection and inflammation can cause CRS that occasionally can give rise to polyp growth or if these two entities develop independently of each other. However, the present finding of a high, steroid-dependent NLR expression in nasal polyps and an infection-independent NLR expression in tonsils suggests that NLRs might have a specific role in the polyp development.

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Conflict of interest

The authors confirm that there are no conflicts of interest.

Author contribution

AM performed most of the experiments, analyzed the data, and wrote the paper. JB took part in data analysis and immunohistochemistry. AC provided nasal polyps and RU helped with immunohistochemistry. LOC critically revised the manuscript and provided financial support. All authors have read and approved the final form of the manuscript.

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