

## Original article

# Human $\beta$ -defensins and toll-like receptors in the upper airway

**Background:** Measurement of innate markers in nasal mucosa, tonsils and adenoids might lead to new views about the role of innate immunity in the upper airway. In this study, the expression of human  $\beta$ -defensins (HBD) 2 and 3 and toll-like receptors (TLR) 2 and 4 in various upper airway diseases was investigated.

**Methods:** Surgical samples from patients with tonsillar disease ( $n = 18$ ), hypertrophic adenoids ( $n = 10$ ) and sinonasal disease ( $n = 30$ ) (chronic sinusitis, nasal polyps, turbinate mucosa as controls) were investigated by immunohistochemistry. Quantification of HBD-2 and 3 mRNA, TLR-2 and 4 mRNA expression was performed by real-time polymerase chain reaction (PCR).

**Results:** Immunohistochemistry revealed a strong expression of HBD-2 in tonsillar tissue. Quantification of HBD-2 and HBD-3 mRNA showed a more than tenfold higher expression in tonsillar tissue than in adenoids, whereas in nasal biopsies, only negligible defensin expression could be measured. No significant differences were found for TLR-4 between the various tissues, whereas TLR-2 expression in adenoids was significantly lower compared with other tissues.

**Conclusion:** These results demonstrate a strong defensin expression in tonsillar tissue compared with nasal and paranasal mucosa and adenoids. Toll-like receptor expression in all these tissues illustrates a possibly important immunological sentinel function of upper airway mucosa.

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Key words: adenoids; chronic sinusitis;  $\beta$ -defensins; innate immunity; lymphoid tissue; nasal polyps; polymerase chain reaction; toll-like receptor; tonsils.

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The upper airway has an important role in the first encounter of antigens and pathogens with the body. It is well known that adaptive immunity functions through specialized structures like tonsils and adenoids, with a characteristic mucosal-lymphoid association [nasal associated lymphoid tissue (NALT)] (1). Also the delicate undifferentiated epithelial lining of the nose has developed several mechanisms for protection against microorganisms. These are unspecific mechanical factors such as mucociliary clearance, triggering of sneezing and surface phagocytic clearance, contributing to the prevention of microorganism invasion (2, 3). Antimicrobial peptides, of which the  $\beta$ -defensins are constitutively or inducibly expressed in human epithelia and mucosal surfaces, co-operate with these mechanical factors to give an immediate protection against bacterial overgrowth (4–6). Defensins are bactericidal and fungicidal peptides on the body surfaces of invertebrates and vertebrates and in phagolysosomes of circulating phagocytes. Defensins can also induce and enhance an appropriate local and systemic adaptive answer through their signalling function (7).

Human  $\beta$ -defensin (HBD)-2 exhibits a potent antimicrobial activity against gram-negative bacteria (*Escheria coli*, *Pseudomonas aeruginosa*) and *Candida*, and only a bacteriostatic activity against *Staphylococcus aureus* (8). HBD-3 shows bactericidal activity against gram-negative as well as gram-positive bacteria, including *S. aureus*, probably because of its capacity to form an amphipathic dimer structure and its increased positive surface charge compared with HBD-2 (8). In contrast to HBD-1, which is constitutively expressed in epithelial surfaces (9), HBD-2 and HBD-3 expression is more variable and inducible (8). HBD-2 represents the first human defensin shown to be produced following stimulation of epithelial cells with microorganisms (gram-negative, gram-positive bacteria and *Candida Albicans*) or cytokines such as tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) (8). HBD-3 expression is induced by the same external stimuli, but also upon stimulation with interferon (IFN)- $\gamma$  (10, 11).

The promotor regions of HBD-2 genes contain several sequences recognized transcription factors, including NF- $\kappa$  B (12–14). Upstream of the coding sequence of HBD-3, there are no NF- $\kappa$  B response elements suggesting that the expression of HBD-2 and HBD-3 genes is differently regulated (15, 16). The HBD-2 and HBD-3 gene and proteins are locally expressed in inflammatory

**Abbreviations:** HBD, human  $\beta$ -defensin; TLR, toll-like receptor; NALT, nasal associated lymphoid tissue; M cells, membranous epithelial cells; BAL, bronchoalveolar lavage; LPS, lipopolysaccharide.

skin lesions such as psoriasis (8, 17). HBD-2 has been found in the infected lung epithelia of patients with cystic fibrosis (18). Expression of HBD-3 has been localized in heart, skeletal muscle, placenta, oesophagus, trachea, oral mucosa and skin tissues (11, 15).

However, although a specific spectrum of activity and a selective induction mechanism for every defensin has been proposed, the influence of the microenvironment on local defensin expression and production is still poorly understood. Studies of these peptides in tissue samples obtained from patients with different physiopathological influences will elucidate the possible (therapeutic) value of natural innate antimicrobial molecules. In this study, the expression of HBD-2 and HBD-3 mRNA was evaluated in tonsillar tissue in two disease states (idiopathic hypertrophic tonsils *vs* recurrent tonsillitis), in hypertrophic adenoids and in the sinonasal tissue, in chronic sinusitis, nasal polyps and turbinate mucosa.

The internal epithelial surface of the upper airway provides not only a barrier but also site of active interaction between pathogens and the innate and adaptive immune systems. Tonsils and adenoids, which are located at a critical position for immunological detection of airborne or ingested antigens, possess differentiated membranous epithelial cells (M cells) (19). It is suggested that these specialized M cells with their microvillous surface, abundant organelles, micropinocytotic vesicles and close relation to underlying lymphoid cells, play an important role in antigen sampling (20). However, M cells represent only a small percentage (<5%) of follicle associated epithelial cells and, in addition, are not found in nonlymphoid associated mucosa (e.g. epithelial lining of the nose) (21). The ability of monocytes/macrophages or granulocytes to recognize foreign microbial matter is well known since long, however the discovery of toll-like receptors (TLRs), a specific recognition mechanism for microorganisms, on epithelial cells suggests a role for the surface epithelium in the frontline of the mucosal immune system (22, 23). This also suggests a possible recognition by the underlying immune system of antigen without prior damage to the epithelial barrier. TLR expression has been detected on different cells of the immune system (monocytes, macrophages, dendritic cells,  $\gamma\delta$  T cells, Th1 and Th2  $\alpha\beta$  T cells and B cells) illustrating their role in modulating inflammatory responses (24). TLR expression on intestinal epithelial cells (25, 26), human oral epithelial cells (23) and airway epithelia (27) supports their sentinel role at the interface with the environment. Currently, no studies have measured TLR expression in various upper airway tissues (in tonsils, adenoids as well as in nasal mucosa) and in different disease states.

In this study, the role of nasal mucosa and NALT were attempted to determine as sentinels for the innate immunity by the measurement of TLR-2 and TLR-4 expression in tonsillar disease, hypertrophic adenoids and sinonasal disease.

## Material and methods

Tissue samples were collected during surgery and immediately snap frozen in liquid nitrogen.

Three specific tissues were investigated in this study: tonsils, adenoids and nasal mucosa.

In the tonsil group, we distinguished two groups: (1) eight patients (six males, two females, aged 2–5 years) who underwent a tonsillectomy under general anaesthesia because of idiopathic tonsillar hypertrophy (substantial enlarged tonsils, no history of recurrent tonsillitis) and (2) 10 patients (one male, nine females, aged 2–37 years) who underwent a tonsillectomy under general anaesthesia because of recurrent tonsillitis (small or substantial enlarged tonsils, history of recurrent tonsillitis).

In the adenoid group, 10 patients (eight males, two females, aged 2–14 years) were included, who underwent an adenoidectomy because of enlarged adenoids causing nasal obstruction.

In the nasal mucosa group, 30 patients who underwent functional endoscopic sinus surgery were divided into three groups: (1) 10 patients with nasal polyps (five males, five females, aged 21–81 years), (2) 10 patients with chronic sinusitis (five males, five females, aged 23–71 years) and (3) 10 control patients without mucosal disease (inferior turbinate mucosa) (eight males, two females, aged 14–63 years).

## Immunohistochemistry

Cryostat sections (6  $\mu$ m) were prepared and mounted on Super-Frost Plus glass slides (Menzel Glaeser, Braunschweig, Germany). Specimens were fixed in 4% buffered formaldehyde and washed with Phosphate buffer saline (PBS) containing 15% sucrose. Endogenous peroxidase was blocked for 20 min with 0.3%  $H_2O_2$ , 0.1% sodium azide in TBS. Slides were incubated for 30 min in 10% milk powder to block nonspecific binding. For the detection of HBD-2, sections were incubated for 60 min with the primary antibody (goat anti-human HBD-2, Peprtech, London, UK). As negative control, normal goat immunoglobulin G (IgG) (Peprtech) was used. After washing, the expression of HBD-2 was visualized using the LSAB technique conjugated with peroxidase according to the manufacture's instructions (labelled streptavidine-biotin, Dako Cytomation, Heverlee, Belgium). Sections were incubated with AEC-chromogen, which results in a red-stained precipitate. Finally, sections were counterstained with haematoxylin and mounted.

## RNA preparation and real-time-quantitative RT-PCR

Snap frozen tissue samples were placed in liquid nitrogen and thoroughly grinded with a mortar and pestle and homogenized using QIAshredder (Qiagen, Hilden, Germany). Total RNA was purified using RNeasy (Qiagen) as recommended by the manufacture. Total RNA of 1  $\mu$ g was reverse transcribed to generate cDNA with SuperScript<sup>TM</sup> II (Invitrogen, Merelbeke, Belgium) in the presence of oligo(dT), random primer, DTT and dNTPs.

Primers and probes used for polymerase chain reaction (PCR) amplification were purchased from Eurogentec (Liège, Belgium) and sequences are listed in Table 1.

Real-time PCR was performed in a 25  $\mu$ l reactions mixture [composed of 5  $\mu$ l cDNA (equivalent to 25 ng total RNA), 0.4  $\mu$ M (final concentration) oligonucleotide primers, 0.1  $\mu$ M probe, 5 mM  $MgCl_2$ , 200  $\mu$ M dNTPs, and 0.025 U/ $\mu$ l Hot GoldStar enzyme (Eurogentec)] measured with a Perkin Elmer ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Reactions were incubated for 10 min at 95°C, followed by 45 cycles of a two-step amplification-procedure composed of annealing/extension at 60°C for 1 min and denaturation for 15 s at 95°C. In

Table 1. Sequences of primers and probes used for PCR amplification

|                                                      |
|------------------------------------------------------|
| (1) Human HBD-2                                      |
| Sense, 5'-TGATGCCTCTCCAGGTGTTT-3';                   |
| Antisense, 5'-GGATGACATATGGCTCCACTCTT-3';            |
| Probe, 5'-6FAM-TGGTGGTATAGGCGATCCTGTTACCTGC-TAMRA-3' |
| (2) Human HBD-3                                      |
| Sense, 5'-TATCTTCTGTTTGGCTTTGCTCTTCC-3';             |
| Antisense, 5'-CCTCTGACTCTGCAATAATTTCTGTAAT-3';       |
| Probe, 5'-6FAM-TTGGTGCCTGTCCAGGTCATGGAG-TAMRA-3'     |
| (3) Human TLR-2                                      |
| Sense, 5'-GGCCAGCAAATTACCTGTGTG-3';                  |
| Antisense, 5'-AGGCGGACATCCTGAACCT-3';                |
| Probe, 5'-6FAM-CTCCATCCCATGTGCGTGGC-TAMRA-3'         |
| (4) Human TLR-4                                      |
| Sense, 5'-CTGCAATGGATCAAGGACCA-3';                   |
| Antisense, 5'-TTATCTGAAGGTGTTGCACATTCC-3';           |
| Probe, 5'-6FAM-AGGCAGCTCTTGGTGGAAGTTGAAC-TAMRA-3'    |
| (5) PBGD                                             |
| Sense, 5'-GGCAATGCGGCTGCAA-3';                       |
| Antisense, 5'-GGGTACCCACGCGAATCAC-3';                |
| Probe, 5'-6FAM-CATCTTTGGGCTGTTTCTTCCGCT-TAMRA-3'     |

each run, dilution series of pooled cDNA from donor adenoids were amplified to serve as a standard curve for calculation of relative quantities.

Statistical analysis

Data are expressed as median and interquartile range (IQR). When comparisons were made between groups, significant between-group variability was first established using the Kruskal–Wallis test. The Mann–Whitney *U*-test was then used for between-group (unpaired) comparison. Spearman rank correlation coefficient (*r*) was used to assess the relationships between the parameters. *P*-values less than 0.05 were considered statistically significant.

Results

HBD-2 and HBD-3

Immunohistochemistry demonstrated staining for HBD-2 peptide in most tonsils, both in the surface epithelium and in the crypt epithelium of the tonsil (Fig. 1). In adenoids, HBD-2 staining could be detected only in two selected cases which had borderline positive HBD-2 mRNA expression. In the mucosa of the turbinates and in patients with chronic sinusitis or nasal polypsis, immunohistochemistry remained negative for HBD-2.

Quantification of HBD-2 and HBD-3 expression by real-time PCR confirmed a strong expression of HBD-2 and HBD-3 mRNA in tonsillar tissue in with no significant difference between the idiopathic hypertrophic tonsillar disease and recurrent tonsillitis. Furthermore, HBD-2 and HBD-3 showed a significant correlation in mRNA expression in tonsillar disease (*r* = 0.64; *P* = 0.002). HBD-2 and HBD-3 mRNA was below detection level in adenoids and in sinonasal mucosa of patients with chronic sinusitis, nasal polyps or turbinate mucosa of controls. Taken together, HBD-2 and HBD-3 was significantly more expressed (*P* = 0.02; *P* < 0.001,

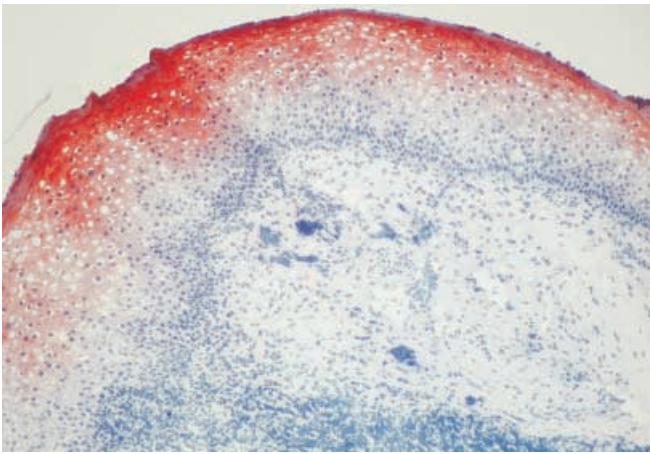


Figure 1. Staining for HBD-2 in tonsils (original magnification 100x): surface epithelium.

respectively) in tonsillar tissue compared with adenoids and sinonasal mucosa (Fig. 2).

TLR-2 and TLR-4

Real time PCR showed a constant expression of TLR-2 and TLR-4 mRNA in all tissues examined. In tonsillar disease, there was no significant difference of expression for both TLRs between the different patient groups. A statistically significant (*P* = 0.04) lower expression of TLR-2 in adenoids was measured compared with all tonsillar tissue. TLR-2 and TLR-4 mRNA expression in sinonasal mucosa was clearly shown, but no difference in the selected patient groups could be assessed. Yet, TLR-2 and TLR-4 mRNA expression in nasal tissues were significantly correlated (*r* = 0.57, *P* = 0.001) (Fig 3).

A correlated expression for defensins and TLR mRNA was difficult to assess, yet we measured a significant inverse correlation between HBD-3 and TLR-4 (*r* = -0.517; *P* = 0.02) in tonsillar tissue.

Discussion

The concept, that the mucosa of the respiratory tract acts as an immunological organ is now widely accepted. It was proposed that in the airway epithelia both HBD-2 and HBD-3 respond to pro-inflammatory stimuli with an increase in mRNA expression and peptide abundance (9, 28). It has also been demonstrated that HBD-2 is present in higher levels in bronchoalveolar lavage (BAL) or plasma specimens from patients with inflammatory lung disease such as cystic fibrosis (28), pneumonia (29) and atypical mycobacterial infections (30) than in healthy persons.

We have shown here that in sinus disease, the environmental triggering was apparently not sufficient to induce HBD-2 and HBD-3 expression or production. There was no baseline detection of HBD-2 and HBD-3 in

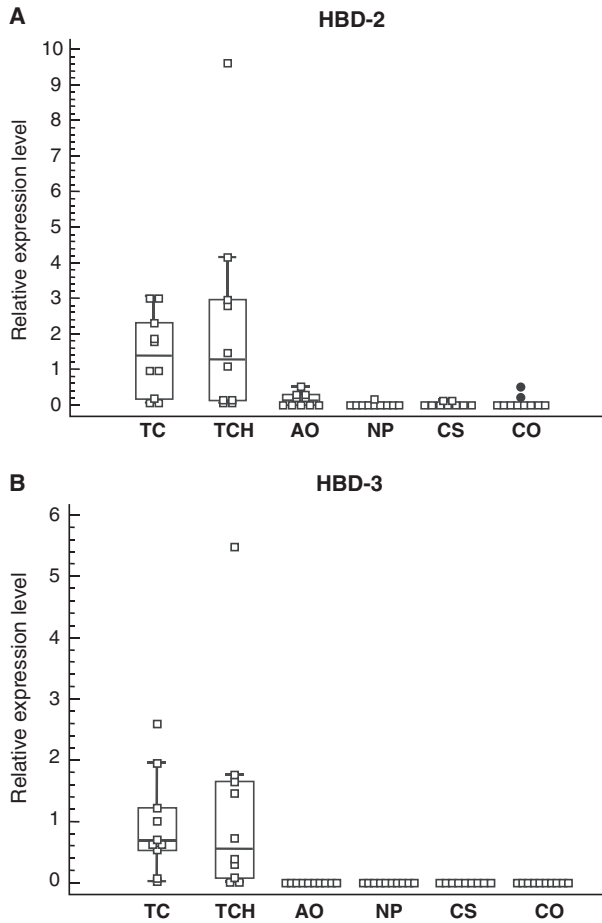


**Figure 2.** Quantification of HBD-2 (A) and HBD-3 (B) with real-time PCR (box plot graph) TC: idiopathic tonsillar hypertrophy, TCH: chronic tonsillitis, AO: obstructive adenoid hypertrophy; NP: nasal polyposis, CS: chronic sinusitis, CO: controls (inferior turbinate) y-axis: R.E.L. (relative expression level) (arbitrary unit).

any sinus sample and no upregulation could be measured for HBD-2 and HBD-3 in paranasal mucosa in patients with chronic sinusitis or nasal polyposis compared with turbinate mucosa of the control group.

In human palatine tonsils the expression of HBD-2 has been shown before (31). We tried to establish whether the disease state of the tonsil was related to the expression of HBD-2. No significant difference in HBD-2 mRNA expression could be established between both patient groups (idiopathic hypertrophic tonsils vs recurrent tonsillitis). Furthermore, the absence of HBD-2 and HBD-3 mRNA in adenoids and (para)nasal mucosa emphasizes that normal bacterial triggering in this tissue is insufficient to stimulate defensin expression and production or that other defence mechanisms take over this task.

Possibly, the intensive mechanical clearance in the nose and nasopharynx helps to diminish the contact time with environmental microorganisms, which could mean that defensin induction needs a more intensive and prolonged

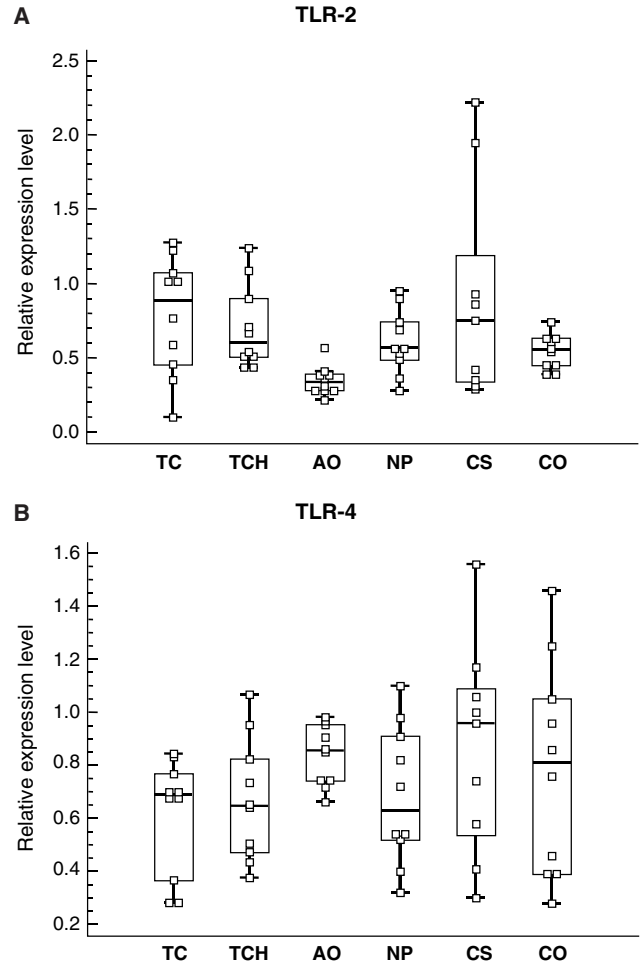


**Figure 3.** Quantification of TLR-2 (A) and TLR-3 (B) with real-time PCR (box plot graph) TC: idiopathic tonsillar hypertrophy, TCH: chronic tonsillitis, AO: obstructive adenoid hypertrophy; NP: nasal polyposis, CS: chronic sinusitis, CO: controls (inferior turbinate) y-axis: R.E.L. (relative expression level) (ration gene expression/housekeeping gene expression).

microorganical triggering. The deep crypts and the excessive microorganical load and bacterial diversity in tonsils (32) could explain the more pronounced presence of inducible antimicrobial peptides in this tissue.

The ancient co-existence between micro- and multi-organical lives probably made it possible to coexist without exhausting defense resources. In physiologic circumstances, innate defense mechanisms might be less important, and its fast, powerful and specific defense mechanisms are preserved until the mechanical barrier function fails. Singh et al. (9) suggested that airway epithelia might wait for a second response signal, such as IL-1 release from macrophages, before responding by inducing expression and secretion of HBD-2. Indeed, they found that the induction of the HBD-2 gene expression in well-differentiated primary cultures of human airway epithelia was much more responsive to lipopolysaccharide (LPS) when the epithelia were exposed to bacterial products in the

presence of alveolar macrophages. Additional research is needed to determine the relative importance of multiple bacterial products and cytokines in the direct regulation of  $\beta$ -defensin expression in airway epithelia.

Another question remains: what are the relevant receptors and ligands that determine the expression of antimicrobial peptides in human?

Common molecular repeating patterns on pathogens are recognized directly by pattern-recognizing receptors (PRRs) like TLRs and a cellular response is induced mediated by a transmembrane signal transduction (33). Triggering of the intracellular signalling pathway of TLRs does not only initiate innate immunity responses, it can also lead to the induction of adaptive immunity because of induction of cytokines, chemokines and co-stimulatory molecules (CD80 and CD86 on macrophages and dendritic cells) (34). Currently 10 distinct TLRs have been described, each with different binding capacity. Both TLR-2 and TLR-4 have been identified as signalling receptors activated by bacterial cell wall components (35). TLR-4 signals the presence of LPS by associating with CD14 (14) in macrophages, while TLR-2 signals the presence of different microbial constituents like proteoglycans or lipoteichoic acid of the gram-positive bacteria (36, 37). Therefore, it has been suggested that the expression of TLR 2 confers responsiveness to *S. aureus* and *Streptococcus pneumoniae*, gram-positive bacteria of important clinical significance in upper airway disease and that TLR-4 may be more specific for gram-negative bacteria (38, 39). In our investigation with real-time PCR, a clear expression of TLR-2 and TLR-4 mRNA in all tissues was shown. Ligand binding of TLRs induces signal transduction via NF- $\kappa$ B dependent pathways (39) and genomic analysis of the 5' flanking region of the  $\beta$ -defensin gene shows consensus sequence sites for regulators of transcription like NF- $\kappa$ B (13). This might imply a role for microbial stimulation via TLRs in  $\beta$ -defensin expression but the exact involvement of TLR ligand binding in  $\beta$ -defensin regulation in human remains to be determined (40). In our study, no correlation between  $\beta$ -defensin and TLR expression could be demonstrated.

However, we show an *in vivo* correlation between TLR-2 and TLR-4 expression in all tissue evaluated, which is in corroboration with Kawai and coworkers (41), who mention the *in vitro* induction of TLR-4 in epidermal

keratinocytes by stimulation with peptoglycan from gram-positive bacteria, suggesting TLR-4 expression regulation through TLR-2 ligand binding. *In vitro* it has also been shown in human monocytes that mRNA of TLR-2 and TLR-4 were downregulated by the same cytokine (IL-4), whereas IL-10 and glucocorticoid dexamethasone, also deactivators of human monocytes, respectively, did not influence and up-regulated TLR 2 and TLR 4 expression (42). This suggests a parallel regulation of TLR 2 and TLR 4.

We found no significant difference of expression for both TLRs between the different patient groups in tonsillar and sinonasal disease. Apparently, microbial or inflammatory triggering in these upper airway conditions provokes the same TLR-2 or TLR-4 expression pattern. With the knowledge that triggering of the innate immune response can present a possible risk for the human organisms (e.g. toxic shock syndrome) (42), it is reasonable to presume that only invasive pathogens, the stimulation by microbial components simultaneously or certain inflammatory conditions (43) could set off the innate response by influencing the TLR-expression. Further studies are needed to determine regulation mechanisms of TLR to better understand innate immunity mechanisms in pathological conditions.

## Conclusion

In this study, we showed at least indirect evidence for the active participation of upper airway epithelium and mucosal-lymphoid tissue in innate host defence. Although the exact mechanism of regulation and induction of both, human defensins and TLRs remains to be elucidated, we can conclude that inducible defensin production is concentrated in lymphoid tissue rather than in airway mucosa. Toll-like receptor expression in nasal mucosa, adenoids and tonsils, illustrates an important immunological sentinel function of upper airway mucosa.

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## References

1. WONG DT, OGRA PL. Immunology of tonsils and adenoids-an update. *Int J Pediatr Otorhinolaryngol* 1980;**2**:181-191.
2. LUND VJ. Nasal physiology: neurochemical receptors, nasal cycle and ciliary action. *Allergy Asthma Proc* 1996;**17**:179-184.
3. KALINER MA. Human nasal host defense and sinusitis. *J Allergy Clin Immunol* 1992;**30**:424-430.
4. COLE A, DEWAN P, GANZ T. Innate antimicrobial activity of nasal secretions. *Inf immune* 1999;**67**:3267-3275.
5. LEE SH, KIM JE, LIM HH. Antimicrobial defensin peptides of the human nasal mucosa. *Ann Otol Rhinol laryngol* 2002;**111**:135-141.
6. GANZ T, LEHRER RI. Defensins. *Curr Opin Immunol* 1994;**6**:584-589.
7. YANG D, CHERTOV O, OPPENHEIM JJ. The role of mammalian antimicrobial peptides and proteins in awakening of innate host defense and adaptive immunity. *CMLS* 2001;**58**:978-989.

8. SCHIBLI DJ, HUNTER HN, AEYEV V, STARNER TD, WIENCEK JM, MCCRAY PB et al. The solution structures of human  $\beta$ -defensin 3 lead to better understanding of the potent bactericidal activity of HBD-3 against *Staphylococcus aureus*. *J Biol Chem* 2002;**277**:8279–8289.
9. SINGH PK, JIA HP, WILES K, HESSELBERTH J, LIU L, CONAWAY BAD et al. Production of  $\beta$ -defensin by human airway epithelia. *Proc Natl Acad Sci* 1998;**95**:14961–66.
10. HARDER J, BARTELS J, CHRISTOPHERS E, SCHRODER JM. Isolation and characterization of Human  $\beta$  defensin 3, a novel human inducible peptide antibiotic. *J Biol Chem* 2001;**276**:5707–5713.
11. GARCIA JR, JAUMANN F, SCHULZ S, KRAUSE A, RODRIGUEZ-JIMENEZ J, FORSSMANN U et al. Identification of a novel multifunctional beta-defensin (HBD-3) with specific antimicrobial activity. Its interaction with plasma membranes of *Xenopus* oocytes and the induction of macrophage chemoattraction. *Cell Tissue Res* 2001;**306**:257–264.
12. HARDER J, BARTELS J, CHRISTOPHERS E, SCHRODER JM. A peptide antibiotic from human skin. *Nature* 1997;**387**:861–862.
13. LUI L, WANG L, JIA HP, ZHAO C, HENG HHQ, SXHUTTE BC et al. Structure and mapping of the human  $\beta$ -defensin gene and its expression at sites of inflammation. *Gene* 1998;**222**:237–244.
14. BECKER MN, DIAMOND G, VERGHESE MW, RANDELL SH. CD14-dependent lipopolysaccharide-induced  $\beta$ -defensin-2 expression in human tracheobronchial epithelium. *J Biol Chem* 2000;**275**:29731–29736.
15. JIA HP, SCHUTTE BC, SCHUDY A, LINZMEIER R, GUTHMILLER JM, JOHNSON GK et al. Discovery of a new human beta defensins using genomics-based approach. *Gene* 2001;**263**:211–218.
16. SCHUTTE BC, MC CRAY PB Jr.  $\beta$ -defensin in lung host defense. *Annu Rev Physiol* 2002;**64**:709–748.
17. ALI RS, FALCONER A, IKRAM M, BISSETT CA, CERIO R, QUINN AG. Expression of peptide antibiotic human  $\beta$  defensin-1 and human  $\beta$  defensin-2 in normal human skin. *J Invest Dermatol* 2001;**117**:106–111.
18. SCHRODER JM, HARDER J. Human beta-defensin-2. *Int J Biochem Cell Biol* 1999;**31**:645–651.
19. CLAEYS S, CUVELIER C, QUATACKER J, VAN CAUWENBERGE P. Ultrastructural investigation of M-cells and lympho-epithelial contacts in naso-pharyngeal associated lymphoid tissue (NALT). *Acta Otolaryngol Suppl* 1996;**523**:40–42.
20. KRAEHEBUHL JP, NEUTRA MR. Epithelial M cells: differentiation and function. *Annu Rev Cell Dev Biol* 2000;**16**:301–332.
21. NEUTRA MR, MANTIS NJ, KRAEHEBUHL JP. Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nat Immunol* 2001;**2**:1004–1009.
22. UEHARA A, SUGAWARA S, TAKADA H. Priming of human oral epithelial cells by interferon- $\gamma$  to secrete cytokines in response to lipopolysaccharides, lipoteichoic acids and peptoglycans. *J Med Microbiol* 2002;**51**:626–634.
23. ASAI Y, OHYAMA Y, GEN K, OGAWA T. Bacterial fimbriae and their peptides activate human gingival epithelial cells through Toll-like receptor 2. *Infect Immun* 2001;**69**:7387–7395.
24. KRUTZIK SR, SIELING PA, MODLIN RL. The role of toll-like receptors in host defense against microbial infection. *Curr Opin Immunol* 2001;**13**:104–108.
25. CARIO E, ROSENBERG IM, BRANDWEIN SL, BECK PL, REINECKER HC, PODOLSKY DK. Lipopolysaccharide activates distinct signaling pathways in intestinal epithelial cell lines expressing toll-like receptors. *J Immunol* 2000;**164**:966–972.
26. CARIO E, PODOLSKY DK. Differential alteration in intestinal epithelial cell expression of toll-like receptor 3 (TLR 3) and TLR 4 in inflammatory bowel disease. *Infect Immunol* 2000;**68**:7010–7017.
27. WANG X, MOSER C, LOUBOUTIN JP, LYSSENKO AS, WEINER DJ, WEISER JN, WILSON JM. Toll-like receptor 4 mediates innate immune responses to *Haemophilus influenzae* infection in mouse lung. *J Immunol* 2002;**168**:810–815.
28. HARDER J, MEYER-HOFFERT U, TERAN LM, SCHWICHTENBERG I, BARTELS J, MAUNE S, SCHRODER JM. Mucoid *Pseudomonas aeruginosa*, TNF- $\alpha$ , and IL-1 $\beta$ , but not IL-6, induce human beta-defensin-2 in respiratory epithelia. *Am J Respir Cell Mol Biol* 2000;**22**:714–721.
29. HIRATSUKA T, NAKAZATO M, DATE Y, ASHITANI J, MINERNTSU T, CHINO N, MATSUKURA S. Identification of human beta-defensin-2 in respiratory tract and plasma and its increase in bacterial pneumonia. *Biochem Biophys Res Commun* 1998;**249**:943–947.
30. ASHITTANI J, MUKAE H, HIRATSUKA T, NAKAZATO M, KUMAMOTO K, MATSUKURA S. Plasma and BAL fluid concentrations of antimicrobial peptides in patients with *Mycobacterium Avium*-intracellular infection. *Chest* 2001;**119**:1131–1137.
31. WEISE JB, MEYER JE, HELMER H, WITROCK H, MAUNE. A newly discovered function of palatine tonsils in immune defence: the expression of defensins. *Otolaryngol Pol* 2002;**56**:409–413.
32. BROOK I, YOCUM P, SHAH K. Surface versus core-tonsillar aerobic and anaerobic flora in recurrent tonsillitis. *JAMA* 1980;**244**:1696–1698.
33. MEDZHITOV R, JANEWAY CA Jr. Innate immune recognition: mechanisms and pathways. *Immunol Rev* 2000;**173**:89–97.
34. JANEWAY CA Jr, MEDZHITOV R. Innate immune recognition. *Annu Rev Immunol* 2002;**20**:197–216.
35. LIEN E, INGALLS RR. Toll-like receptors. *Crit Care Med* 2002;**30**:S1–S11.
36. AKIRA S, TAKEDA K, KAISHO T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001;**2**:675–680.
37. SCHWANDNER R, DZIARSKI R, WESCHE H, ROTHE M, KIRSCHNING CJ. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by toll-like receptor 2. *J Biol Chem* 1999;**274**:17406–17409.
38. LAFLAMME N, RIVEST S. Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. *FASEB J* 2001;**15**:155–163.
39. SHUTO T, XU H, WANG B. Activation of NF- $\kappa$ B by nontypeable *Haemophilus influenzae* is mediated by toll-like receptor 2-TAK1-dependent NIK-IKK $\alpha$ /I $\kappa$ B $\alpha$  and MKK3/6-p38 MAP kinase signaling pathways in epithelial cells. *PNAS* 2001;**98**:8774–8779.
40. DIAMOND G, RUSSELL JP, BEVINS CL. Inducible expression of an antibiotic peptide gene in lipopolysaccharide-challenged tracheal epithelial cells. *Proc Natl Acad Sci* 1996;**93**:5156–5160.
41. KAWAI K, SHIMURA H, MINAGAWA M, ITO A, TOMIYAMA K, ITO M. Expression of functional toll-like receptor 2 on human epidermal keratinocytes. *J Dermatol Sci* 2002;**30**:185–194.
42. BEUTLER E, GELBART T, WEST C. Synergy between TLR-2 and TLR-4: a safety mechanism. *Blood Cells Mol Dis* 2001;**27**:728–730.
43. HAUSMANN M, KIESSLING S, MESTERMANN S, ROGGER G. Toll-like receptors 2 and 4 are upregulated during intestinal inflammation. *Gastroenterology* 2002;**122**:1987–2000.