

Original article

Macrophage mannose receptor in chronic sinus disease

Background: The role of infectious agents in the onset and maintenance of chronic sinus disease is still not fully understood. Macrophage mannose receptor (MMR), an innate pattern recognizing receptor, capable of phagocytosis of invaders and signal transduction for proinflammatory mechanisms, might be of importance in immune interactions in chronic sinus disease.

Objective: We examined the MMR in sinonasal airway mucosa to evaluate its possible role in chronic rhinosinusitis (CS) and nasal polyposis (NPs).

Methods: Surgical samples from patients with sinonasal disease were investigated with real-time RT-PCR for quantification of MMR mRNA expression, and the presence and location of MMR-positive cells was analysed by immunohistochemistry.

Results: Quantification of MMR mRNA showed a statistically significant higher expression in NPs compared to CS without NP and controls. Immunohistochemistry revealed expression of MMR in all tissue samples; however, in NP we found an enhanced positive cellular staining including cell aggregates.

Conclusions: We could demonstrate for the first time that the expression of MMR is significantly upregulated in NP compared to patients with CS without NP or turbinate tissue of controls. Macrophages expressing MMR, accumulated in cell aggregates in NPs, play a possible key role in pathogen–macrophage interaction in NP disease.

**S. Claeys¹, T. De Belder¹,
G. Holtappels¹, P. Gevaert¹,
B. Verhasselt², P. Van Cauwenberge¹,
C. Bachert¹**

¹Department of Otorhinolaryngology; ²Department of Clinical Chemistry, Microbiology and Immunology, Ghent University, Ghent, Belgium

Key words: chronic sinusitis; immunohistochemistry; innate immunity; macrophages; mannose receptor; nasal polyps; real time RT-PCR.

Sofie Claeys
Department of Otorhinolaryngology
University Hospital
De Pintelaan 185
B-9000 Ghent
Belgium

Accepted for publication 12 November 2003

Nasal polyposis (NPs) is a chronic disease of the nose and sinuses with severe predominantly eosinophilic tissue (1) inflammation and a high recurrence rate after medical or surgical treatment (2), often leading to a substantial impairment of the quality of life of the patient (3). Nasal polyps may specifically occur in patients with cystic fibrosis, primary ciliary dyskinesia or allergic fungal sinusitis. In the general population, however, the prevalence of NPs ranges from 1 to 4% and the precise mechanism underlying the pathogenesis of NPs is unknown and probably multifactorial. The predominant type of nasal polyp disease is bilateral, eosinophilic and frequently linked to asthma and aspirin intolerance (4, 5). In previous investigations we showed a significantly higher concentration of IL-5, eotaxin, ECP, and leukotrienes (LT) C4/D4/E4 (6), in nasal polyp tissue compared to nonpolyp sinus tissue. Specific IgE to *Staphylococcus aureus* enterotoxins and polyclonal IgE formation is found to be increased in nasal polyp tissue and correlates with markers of eosinophilic inflammation, which points to a possible role of bacterial superantigens in the pathophysiology of NPs (5). In contrast, no IgE

antibodies to enterotoxins have been found in chronic sinusitis (CS) (unpublished data).

Micro-organisms that cross the epithelial barrier are almost immediately recognized by macrophages that reside in the tissue of the host. Macrophages, which mature continuously from circulating monocytes leaving the circulation, migrate in large numbers into the connective tissue. In the submucosal lining of the gastrointestinal and respiratory tracts these phagocytotic cells have a key role in innate immunity because they can recognize, ingest and destroy many pathogens without support of the adaptive immune response.

The mannose receptor, a pattern recognizing innate receptor (PRR) expressed on subsets of macrophages, immature dendritic cells and a population of endothelial cells is a key molecule in antigen recognition (7, 8). The macrophage mannose receptor (MMR) is a 175-kDa transmembrane glycoprotein containing three types of characterizing domains, two of which have distinct carbohydrate recognizing properties. The amino-terminal cysteine-rich domain plays a critical role in binding sulphated glycoproteins. The C-type lectin domains facilitate carbohydrate-dependent uptake of mannosylated protein antigens on micro-organisms including bacteria, yeast, enveloped viruses and protozoans (9). The MMR shows high affinity for mannose and fucose, intermediate

Abbreviations: CS, chronic sinusitis; MMR, macrophage mannose receptor; NP, nasal polyposis; PRR, pattern recognizing receptor; TLR, toll-like receptor.

affinity for *N*-acetylglucosamide and glucose and low affinity for galactose (10). Because these terminal sugars are rarely found on mammalian cell-surface, the MMR could be responsible for recognition of self and nonself antigens (11). It also plays a key role in pathogen-related acquired host defence by mediating antigen internalization and delivery to MHC class II compartments for antigen presentation (11, 12).

The MMR is not only responsible for immediate recognition and elimination of pathogens but also functions as a signal transducing receptor, triggering cytokine production (13, 14) and modulating cell surface receptors (15–17). MMR expression and the functional capacity of this receptor is modulated by cytokines (18–24), pathogens and their products (25, 26), but also depends on the phenotype of the macrophage. Indeed, the MMR is not found on circulating monocytes, but is abundantly expressed on differentiated macrophages (10).

Accumulation of macrophages expressing the phagocytotic MMR in inflamed tissue, on the one hand, and the ability of MMR to recognize micro-organisms and to activate secretion of inflammatory products by the MMR–ligand binding, on the other hand, may represent key factors of pathogen–macrophage interactions in chronic upper airway mucosal inflammation. Therefore, we aimed to investigate MMR expression in sinonasal airway disease in patients with CS with and without nasal polyps.

Materials

Sinonasal tissue samples were collected during surgery and immediately snap frozen in liquid nitrogen. Thirty patients who underwent functional endoscopic sinus surgery or septal surgery, for reasons unrelated to the study, were divided into three groups: (i) ten patients with nasal polyps (NPs, five males, five females, 21–81 years); (ii) ten patients with CS without nasal polyps (CS, five males, five females, 23–71 years) and (iii) ten control patients without mucosal disease (inferior turbinate mucosal biopsies) (eight males, two females, 14–63 years).

Chronic sinusitis was diagnosed according to the clinical criteria by Lund and Kennedy (27) on the basis of history, clinical examination, nasal endoscopy and sinus computed tomography scanning. In the CS patient group, no nasal polyps were found by endoscopic evaluation prior or during surgery. Patients with endoscopically diagnosed nasal polyp tissue prior or during surgery were registered in the NP group.

A history of asthma was registered in six patients with NPs, with one subject also demonstrating a history of aspirin sensitivity. None of the patients with CS had a history of asthma or another predisposing disease. Skin prick test was positive for at least one inhalant allergen in three patients with NP and in two patients with CS. Medical management strategy was identical in both sinusitis groups. Three patients with CS and two patients with NP were treated with oral corticoids 6 months prior to surgery. No patient used oral corticoids within the last 4 weeks prior to surgery.

As positive controls we examined MMR expression in tonsillar tissue from 20 patients who underwent tonsillectomy for chronic tonsillitis (28).

The ethical committee of the Ghent University Hospital approved the study, and informed consent was obtained from all subjects prior to inclusion in the study.

Methods

Immunohistochemistry

Cryostat sections (6 µm) were prepared and mounted on Super-Frost Plus glass slides (Menzel Glaeser, Braunschweig, Germany). Specimens were fixed in acetone. Endogenous peroxidase was blocked for 20 min with 0.3% H₂O₂, 0.1% sodium azide in TBS. Specimens were incubated for 1 h with the primary antibody: MMR (clone: 15-2-2, TNO, Leiden, The Netherlands), CD68 (clone: EBM 11, Dako Cytomation, Heverlee, Belgium) and CD1a (clone: NA 1/34, Dako Cytomation). The detection was performed using the LSAB method (labelled streptavidine-biotin, Dako Cytomation) according to the manufacture's instruction. 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 RT-PCR

Snap frozen tissue samples were placed in liquid nitrogen and thoroughly ground with a mortar and pestle and homogenized using QIAshredder (Qiagen, Hilden, Germany). Total RNA was purified using Rneasy (Qiagen) as recommended by the manufacture. One microgram of total RNA was reverse transcribed to generate cDNA with SuperScript™ II (Invitrogen, Merelbeke, Belgium) first strand synthesis kit, used as instructed by the supplier 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) (the sequences are listed in Table 1).

Real-time PCR was performed in a 25-µl reaction mixture [comprising 5 µl cDNA (equivalent to 25 ng total RNA), 10 µM (final concentration) oligonucleotide primers, 2.5 nM probe, 5 mM MgCl₂, 200 µM dNTPs, and 0.025 U/µl Hot GoldStar enzyme (Eurogentec)] measured with a Perkin Elmer ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Reactions were incubated for 10 min at 95°C, followed by 45 cycles of a two-step amplification procedure consisting of annealing/extension at 60°C for 1 min and denaturation for 15 s at 95°C. In each run, dilution series of pooled cDNA from donor adenoids were amplified to serve as a standard curve for the calculation of relative quantities.

Table 1. Sequences of primers and probes used for PCR amplification. PBGD (porphobilinogen deaminase): housekeeping gene, constitutively expressed

Mannose receptor	
Sense:	5'-CAGCGGTTGGCAGTGA-3'
Antisense:	5'-CAGCTGATGGACTTCCTGGTAAG-3'
Probe:	5'-6FAM-TGACCGCAGTCCTTCCGATATTGAAC-TAMRA-3'
PBGD	
Sense:	5'-GGCAATGCGGCTGCAA-3'
Antisense:	5'-GGGTACCCACGCAATCAC-3'
Probe:	5'-6FAM-CATCTTTGGGCTGTTTTCTCCGCT-TAMRA-3'

Statistical analysis

Data are expressed as median and interquartile range. 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. Values of *P* < 0.05 were considered statistically significant.

Results

Expression of MMR mRNA in biopsies obtained during surgery from patients with chronic sinus disease and controls was quantitatively assessed by real-time RT-PCR.

In sinonasal mucosa of patients with CS, the MMR mRNA expression was significantly higher (Fig. 1) in tissue of patients with NPs compared to tissue obtained from CS patients without nasal polyps and inferior turbinate tissue of controls. No statistically significant difference was found between CS and controls (Fig. 1). Furthermore, quantitative evaluation with real-time RT-PCR for MMR mRNA on tonsils from patients with chronic tonsillitis showed a constant expression of MMR mRNA in all samples, at similar levels as in CS and controls (Fig. 1).

Cryostat sections were immunostained to detect and compare the presence and location of the MMR in NPs, CS and controls.

Most nasal tissue samples showed staining for MMR on scattered cells in the subepithelium and lamina propria, although in nonpolyp sinus tissue (Fig. 2) and in turbinate tissue (Fig. 3) staining was low. In contrast, we found perivascular and subepithelial accumulations of MMR-positive cells in NPs (Fig. 4A). Staining of serial slides with CD68 showed accumulation of macrophages matching with the predominant expression of the MMR in nasal polyps (Fig. 4B). These cell aggregates consisted of MMR-positive macrophages, CD3-positive T-cells (Fig. 4C) and CD38-positive plasma cells (Fig. 4D), but lacked staining for CD1a (dendritic cells) and CD20 (B-cells) (data not shown). Immunohistochemical investigations on tonsils revealed scattered MMR expression on cells in the connective tissue and endothelium. However, we could not detect positive

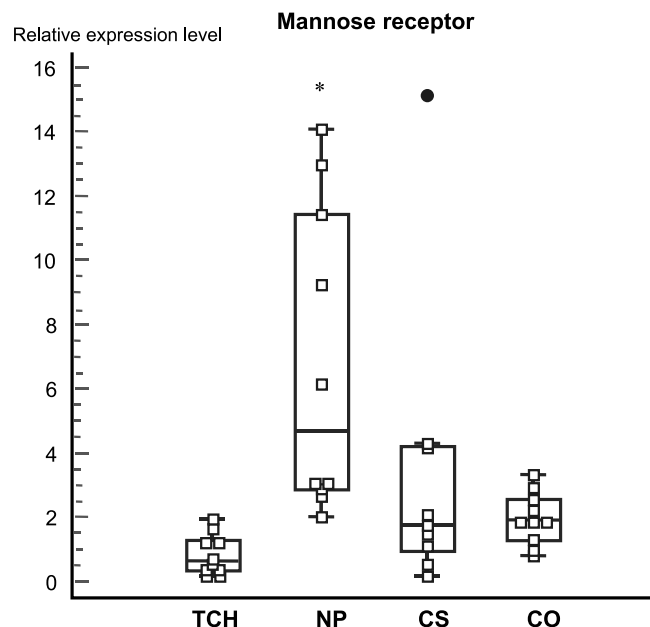


Figure 1. Quantification of MMR mRNA with real-time RT-PCR (box blot graph) in NP, nasalpolyps; CS, chronic sinusitis; CO, controls (inferior turbinate); TCH, chronic tonsillitis. Relative expression level: in relation to PBGD (housekeeping) gene.

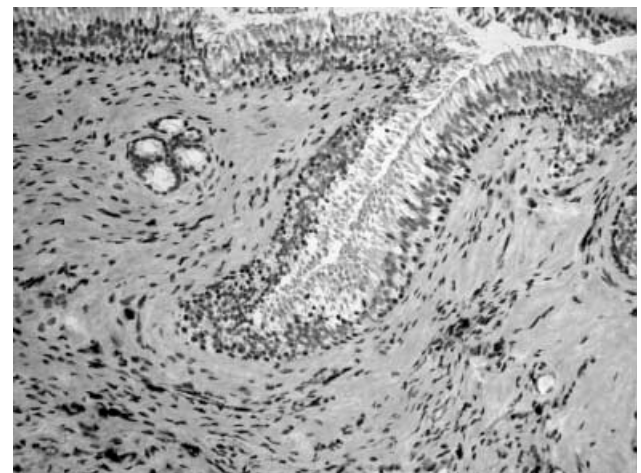


Figure 2. Immunohistochemistry: MMR expression in chronic sinusitis (original magnification 200×).

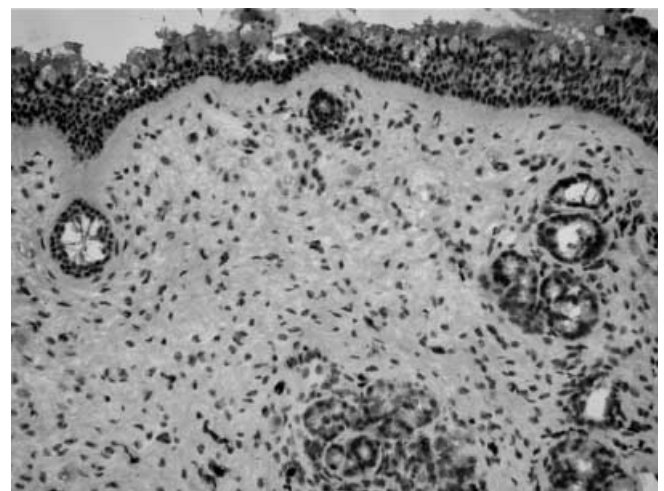


Figure 3. Immunohistochemistry: MMR expression in controls (inferior turbinate) (original magnification 200×).

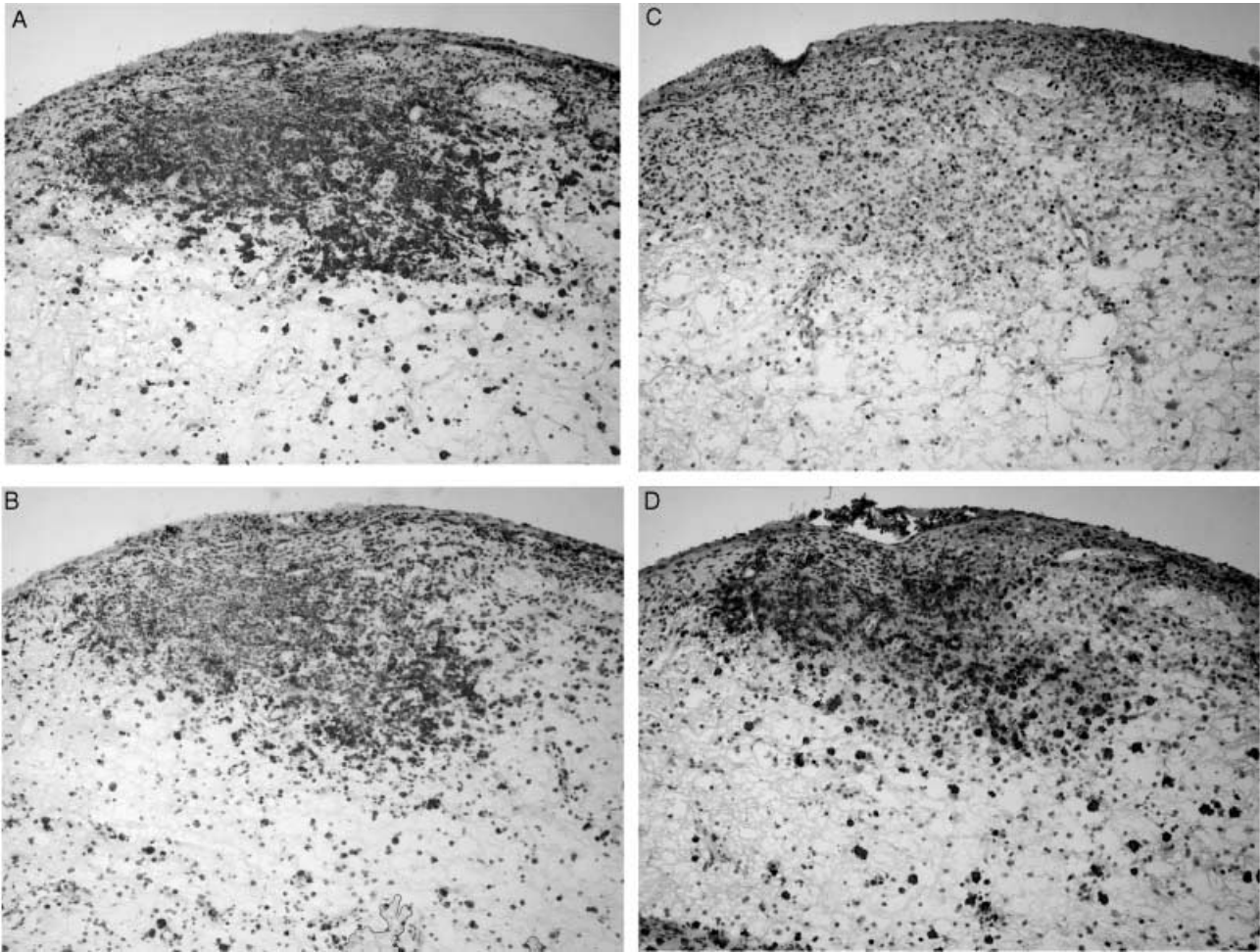


Figure 4. Immunohistochemistry: expression of MMR in nasal polyps (original magnification 200×) (A) is co-localized to CD68 macrophages (B), which form a cell aggregate together with CD3 T-cells (C) and CD38 plasma cells (D).

staining for MMR in the lymphoid follicles of tonsils (data not shown).

Discussion

In this study, quantitative RT-PCR and immunohistochemical staining for MMR expression was performed for the first time in chronic sinus disease.

MMR mRNA expression was significantly higher in NPs compared to CS and controls. Further examination of cryostat sections showed an accumulation of MMR-positive macrophages in the subepithelium of NP tissue, co-localized to T-cells and plasma cells. These findings suggest that the MMR in nasal polyps serves to facilitate phagocytosis of a particular micro-organism by macrophages, leading to T-cell interaction and activation as well as the generation of a humoral immune response.

Although much controversy still exists about the role of viruses, bacteria and fungi in the aetiology of sinusitis, the role of bacteria like *Streptococcus pneumoniae*,

Haemophilus influenzae and *Moraxella catarrhalis* in acute sinusitis is commonly accepted. More conflicting reports are published on the microbiology aspect of CS and the involvement of the normal flora in chronic upper airway disease (29). The cellular events of CS or polyp formation that appear to be the result of a continuous, mainly eosinophil-mediated (30) inflammatory reaction, are so far not directly related to the presence of a single micro-organism. Recently, a potential role for fungi was supposed in CS and NP (31) and in a recent study we investigated nasal tissue from patients with bilateral nasal polyps, which are characterized by a severe eosinophilic inflammation (5), in which we found increased levels of total IgE and *S. aureus* enterotoxin specific IgE antibodies as well as a polyclonal IgE response related to the severity of the eosinophilic inflammation. This suggests that these enterotoxins may act as superantigens in NP. The suggestion of a possible bacterial triggering as a disease modifying mechanism leading to chronic sinonasal inflammation and especially to nasal polyp disease can

also be evaluated through the role of macrophages in these inflammatory processes.

The contribution of macrophages in the regulation of immunological events in mucosal inflammation is evaluated by their role in clearance of pathogens, antigen presentation, lymphocyte activation, tissue repair and their ability to produce inflammatory cytokines and lipid inflammatory mediators (32). Macrophages are capable of rapid recognition and clearance of pathogens, which explains the overall low incidence of clinically significant bacterial infections in the largely colonized respiratory tract, without the initiation of extensive immunological resources. The most thoroughly studied direct mode of recognition, termed lectinophagocytosis, involves the interaction of specific surface glycoconjugates on one cell with corresponding lectins expressed on the other (33). On tissue macrophages, the MMR is probably the most abundant macrophage lectin. This MMR is a PRR on which innate immunity relies to recognize repeated patterns on microbial surfaces and debris. MMR interacts with polysaccharide components of fungal cell walls such as yeast mannan, bacterial cell wall components of Gram-negative and Gram-positive bacteria and lipopolysaccharide.

Although MMR message and proteins were expressed in all tissues examined, in nasal polyp tissue an impressive increase of MMR expression was found compared to controls and CS (Figs 2 and 3). Interestingly, even in samples of chronic tonsillitis as representatives of chronically inflamed lymphatic tissue only baseline expression of MMR was measured (Fig. 1). Previous investigations on other innate PRRs (34) such as toll-like receptor (TLR)-2 and TLR-4 (35) in upper airway tissues showed TLR-2 and TLR-4 mRNA expression in sinonasal mucosa, but no statistically significant difference in expression of mRNA of these receptors in selected patient groups (CS with and without nasal polyps) could be assessed compared to controls.

MMR is so far the only innate PRR known to be significantly upregulated in nasal polyps. These findings suggest a crucial role of MMR in the physiopathology of this form of CS, and differentiate NPs from CS without polyp formation.

In vitro evaluation shows that the expression of MMR on macrophages is dependent on the maturation of the macrophage: freshly isolated monocytes do not express MMR, but expression appears after several days of differentiation (32). The increase of MMR expression in nasal polyp tissue could be due to increased migration of differentiated macrophages into the tissue or due to overexpression of the receptor by resident macrophages. Probably, both mechanisms play a role, but this needs further studies.

Immunohistochemical staining of nasal polyp tissue for MMR added further evidence and showed the accumulation of MMR-positive cells in NP samples but not in CS, chronic tonsillitis or controls.

In NP, the expression of MMR could be matched to macrophages, either as single cells or accumulating to cell aggregates lying in connection with the epithelium. Further analysis of the cell aggregates revealed MMR-positive macrophages co-localized to CD3+ T-cells and plasma cells (Fig. 4C,D), partially IgE positive, but they lacked dendritic cells and B-cells (data not shown). These findings suggest a pathogen-driven immune response, leading to an activation of T-cells and an immunoglobulin switch to IgE without the presence of antigen-presenting cells. The identification of a microbial or molecular substance that may trigger these processes could be of significant therapeutic value. Also, further study on MMR expression regulation in nasal polyp tissue will be needed to determine which mediators influence MMR and macrophage accumulation in this environment. So far the most important cellular difference shown between nasal polyps and CS tissue (without nasal polyps) is the eosinophilic infiltration in NP. No evidence of a higher number of macrophages in nasal polyps compared to CS (without nasal polyps) has been established until now. Surely, the distinction between increased migration of MMR-positive macrophages and the possible upregulation of MMR in different disease states needs to be further investigated. Cytokines in inflammatory tissue [e.g. IL-1 β and TNF- α (32)] may not only prolong survival of recruited monocytes and maintain them in an inflammatory state with an increased potency for releasing inflammatory mediators, but they might also be involved in MMR-expression regulation (32). After ligand binding, the MMR can function as a signal-transducing receptor triggering a variety of responses including secretion of mediators (36), lysosomal enzyme secretion (37), cytokine production (13, 14) and modulation of other cell surface receptors (16–18), which emphasizes the role for MMR as being a link between innate and adaptive immunity. Since the MMR is an innate receptor capable of interaction with pathogen-associated molecular patterns, the increased expression of the MMR in nasal polyps can suggest an increased direct pathogen-macrophage interaction in this tissue. This might be a possible explanation for the onset or maintenance/recurrences described in nasal polyp disease.

The possibility of MMR being an important receptor that initiates/maintains inflammation after binding with otherwise harmless bacteria (nonpathogenic microbes) needs to be further examined. Interactions between innate and adaptive defence pathways need to be further clarified to better understand microbial influences in chronic upper respiratory inflammation.

Conclusions

In this study, MMR tissue expression was demonstrated for the first time in upper airway mucosa. Quantitative real-time RT-PCR revealed statistically significant

increased MMR mRNA expression in nasal polyps compared to CS and was supported by immunohistochemistry, which further revealed MMR-positive macrophage cell aggregates consisting of macrophages, T-lymphocytes and plasma cells in nasal polyp tissue.

These elements suggest a direct interaction between micro-organisms and their products and local immune cells in sinus tissue, illustrating a possible role of MMR-positive macrophages in initiating or maintaining the formation of nasal polyps.

Further investigations are needed to determine the nature of the interaction between innate and adaptive immune responses in this chronic inflammatory disease.

Acknowledgments

This study was supported with grants from the fund Alphonse and Jean Forton and the Belgian Society against Cystic Fibrosis.

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