**Effect of Staphylococcus aureus on the NLRP3 inflammasome, caspase-1 and IL-1β expression in the nasal epithelial cells in chronic rhinosinusitis**

**Abstract**

**Background:** Chronic rhinosinusitis (CRS) is an inflammatory disease. Excessive NLRP3 inflammasome activation and its downstream responses, plays a role in the pathogenesis of CRS.

The context and purpose of the study: The aim of the study was to elucidate the effect of Staphylococcus aureus and budesonide on the mRNA expression and the biologic role (caspase-1 activation and IL-1β secretion) of NLRP3 inflammasome in primary nasal epithelial cells (NECs) in CRS patients and healthy controls.

**Methods:** Brush biopsies isolated from both patients and healthy controls, were denoted respectively for our experiments. These were treated with S. aureus strains (4 strains) only and in combination with budesonide (0, 10, 100, 1000 nM). NECs treated with only budesonide (0, 10, 100, 1000 nM) and untreated NECs were used as controls. Expression of NLRP3, Caspase-1, IL-1β along with NLRC1/2 was analyzed by qPCR. Caspase-1 activity measured by fluorogenic substrates Ac-YVAD-AMC. Enzyme-linked immunosorbent (ELISA) assay performed to measure IL-1β production.

**Results:** The mRNA levels of NLRC1, NLRC2, caspase-1 and IL-1β significantly increased, while NLRP3 demonstrated a trend towards elevation in the CRS group compared to the healthy controls. Infection with S. aureus increased caspase-1 activity and IL-1β secretion. However, treatment with budesonide decreased mRNA expression of NLRC2 and IL-1β secretion.

**Conclusions:** Increase in the caspase-1 activity and IL-1β levels, due to possible activation of NLRP3 inflammasomes, upon S. aureus infection, may have an important role in the pathogenesis of CRS.

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**Introduction**

Chronic rhinosinusitis (CRS) is characterized by persistent inflammation of the nasal and sinus mucous membranes and is often associated with the infection of *Staphylococcus aureus* [1]. Patients with CRS, have 35% carriage rate of *S. aureus* at the middle meatus [2,3] and *S. aureus* biofilms in the sinus mucosa of patients with severe CRS has been reported [4]. Furthermore, the in vivo evidence for the presence of intracellular *S. aureus* in nasal epithelial cells (NECs) obtained from CRS patients, suggests that the intracellular *S. aureus* is a recurrent cause of rhinosinusitis after relapse [5-7].

The epithelial layer in airways has innate immune functions [8,9] and impaired innate immune function is regarded as an etiological factor for pathogenesis of CRS [5]. Airway epithelial cells express pattern recognition receptors (PRRs) such as membrane bound Toll-like receptors (TLRs) and cytosolic nucleotide oligomerisation binding domain (NOD)–like receptors (NLRs) [1,8,9]. PRR detects pathogen-associated
molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). A subgroup of the NLR along with an adaptor protein, and inactive caspase-1 enzyme form an intracellular signaling complex called inflammasome, which is important both for host defense and promoting inflammation [10]. NLRP3 (NOD–like receptor family, pyrin domain containing–3) inflammasome is more versatile as it can be activated by a variety of PAMPs and DAMPs [11]. Inflammasome via activation of caspase-1 can regulate the production of IL-1β, which in turn triggers the acute phase pro-inflammatory responses [12,13]. Excessive activation of the NLRP3 plays a significant role in the chronic airway inflammation, like asthma and chronic obstructive pulmonary disease (COPD) as it exacerbates the disease [14,15]. Caspase-1 is a protease enzyme and plays an important role in the pathogenesis of inflammatory diseases by regulating the cellular export of IL-1β and IL-18 [16].

IL-1β is a key inflammatory mediator driving the host response to infection, injury, and disease produced by innate immune cells in response to PAMPs or DAMPs [17]. Several studies have reported elevated levels of proinflammatory cytokines in the nasal secretions and the sinonasal mucosal tissues in CRS patients [18-20]. Induction of IL-5 and upregulation of IL-8 by Staphylococcus enterotoxin B and S. epidermidis respectively, in nasal polyps have been described [20]. A previous study showed that treatment with a 10-day tapering dose of oral corticosteroids (prednisone) significantly decreased the levels of IL-6 and TNF-α in the sinonasal mucosal biopsy of CRS patients [19]. Although it is already known that NECs express NLRC1, NLRC2, NLRP3 [21], in a recent study it has been shown that NLRP3 and caspase-1 were overexpressed in the NECs from CRS patients with nasal polyps [22]. The activation of the inflammasome in response to pathogen, leads to caspase-1 activation, which in turn, cleaves the precursor IL-1β into two forms: secretory and a biologically active form. Inhibition of IL-1β by an anti-inflammatory drug, is beneficial but the role of corticosteroids as anti-inflammatory drugs on the epithelial cells is contradictory [22]. Inhaled corticosteroids such as budesonide is an anti-inflammatory drug, is beneficial and is used in the treatment of CRS. However, the effect of S. aureus infection and budesonide treatment on the mRNA expression of these NLRs, caspase-1 activity and IL-1β secretion in the NECs remains uncertain. Therefore, the aim of the study was to measure the expression of NLRP3, caspase-1, IL-1β together with NLRC1/2 in vitro in the NECs from healthy donors after S. aureus infection or budesonide treatment. Furthermore, we also aimed to compare the expression of these innate immune mediators in CRS group and healthy controls.

**Materials and Methods**

**Patients and controls**

Nasal brush biopsies were obtained from patients with CRS (n=14). Patients were recruited while undergoing an endoscopic sinus surgery at the Otolaryngology clinic, Örebro University Hospital, Örebro, Sweden during the period of April 2015 - September 2016. The patient group included both the chronic rhinosinusitis with nasal polyps (CRSwNP) patients (n=8) and chronic rhinosinusitis without nasal polyps (CRSsNP) patients (n=6). Healthy volunteers (n=14) were colleagues and staff at the Otolaryngology clinic after exclusion of CRS. All participants in both groups were >18 years. Two ENT specialists (AS and SH) were responsible for the inclusion procedure. The diagnosis of CRS based on history, clinical examination and CT scans according to the European Position Paper on Rhinosinusitis and Nasal Polyps [23]. No specific exclusion criteria were applied. The study was approved by the Uppsala ethical committee (2014/259), all participants gave their informed consent.

**Nasal brush biopsies and primary cell culturing**

Brush biopsies from middle meatus and bulla ethmoidalis (patient group) and from the middle part of the inferior turbinate (control group) were collected using interdental brushes (0.7 mm diameter; Apoteket, Solna, Sweden) and nasal epithelial cells (NECs) were cultured as described previously [24]. Four S. aureus strains (one reference and three clinical isolates), Cowan−1 (reference strain) and 06T023, 06T307, 07T307 (three clinical isolates) were obtained from the Department of microbiology, Örebro University Hospital, Örebro, Sweden. Strains were grown on blood agar plates and incubated at 37°C, overnight. A growth curve experiment performed for all the strains up to 12 hours to determine the log phase (early, mid and late) as no information about bacterial growth curve was available. We determined that these strains would reach their early log phase after 4.5h. Single colony per strain were picked and were grown in Tryptic soy agar broth (TSB) or Luria-Bertani broth (LB) under shaking at 37°C, overnight. Bacterial strains washed using Dulbecco's phosphate-buffered saline (DPBS) by centrifugation at 1000g, 5 min prior to infection experiments.

**Viability assay**

Neutral red uptake assay [25], was performed to measure the viability of primary NECs after infection with S. aureus. For this purpose, the confluent cell monolayer was infected with a multiplicity of infection (MOI) 50MOI Cowan−1, 06T023, 06T307, 07T307 for 2h, 8h and 24h. Cell monolayer exposed to 1% triton X served as positive control.

**Visualization of intracellular S. aureus in NECs**

Fresh S. aureus inoculum was prepared as described previously. S. aureus was re-suspended in DPBS containing 100 μg/ml fluorescein isothiocyanate (FITC; Sigma–Aldrich, Stockholm, Sweden) for 30 min at room temperature. NECs were grown on the collagen coated, rounded 22 mm coverslips in 6 well plates in full–supplemented AECGM (Promo-Cell, Heidelberg, Germany) with added supplements at 37°C in 5% CO2. All experiments were performed with 2–4 passaged cells. NECs were washed three times with DPBS prior to infection with FITC- labeled S. aureus. Cell infection was carried out in medium without antibacterial agents and at MOI 500 for 2.5h. After washing, the infected NECs were fixed with 4% paraformaldehyde and blocked with 5% bovine serum albumin (BSA). S. aureus were stained with 1:500 times diluted anti–Staphylococcus aureus polyclonal primary antibody (ab20920; Abcam, Cambridge, United Kingdom) overnight at 40°C. Cells were washed thrice with DPBS and then incubated with 1:3000...
times diluted CY-5 conjugated–goat polyclonal secondary antibodies (ab6564; Abcam, Cambridge, United Kingdom) and were incubated in the dark for 45 min at room temperature. Cover slips were washed and mounted on glass slides using Vectashield with DAPI (Vector Laboratories Inc., CA, USA). To visualize intracellular and extracellular S. aureus, images were obtained with a 60x immersion lens using BX60 fluorescence microscope (Olympus, Segrate, Italy). Images were analyzed by photo editing software (Adobe Photoshop, Adobe system Inc., CA, USA). Assays were performed with all the strains of S. aureus in triplicates with primary NECs obtained from a healthy control.

**Gene expression analysis**

NECs obtained from healthy controls were used to investigate the effects of S. aureus infection and budesonide treatment on the mRNA expression of innate immune mediators. NECs were infected with 50MOI of S. aureus for 2h or treated with budesonide (0, 10, 100 and 1000 nM) for 24h. After treatment with budesonide supernatants were collected from NECs (for ELISA), cell lysates were used for total RNA extraction by RNA purification kit (Qiagen, Stockholm, Sweden). Total RNA yield was measured by Nanodrop 1000 (Thermofisher scientific, NC, USA) and 500ng RNA was used for subsequent cDNA synthesis using cDNA synthesis kit (Qiagen, Stockholm, Sweden. 7900HT FAST Real–time PCR instrument (Applied Biosystems, Foster City, CA, USA) was used to determine gene expression of target genes. Unlabeled primers and target–specific FAMTM dye labeled TaqMan_ probe (Applied Biosystems, Foster City, CA, USA) specific for NLRC1 (Hs01036727_m1), NLRC2 (Hs01550753_m1), NLRP3 (Hs00198082_m1), Caspase-1 (Hs00354836_m1) and IL-1β (Hs01555410_m1) were used. The comparative quantification algorithms 2ΔΔCt was used to calculate the relative mRNA expression. Value of target gene normalized to corresponding glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (HS 99 99 99 05_m1), and was and fold change calculated in relation to control sample, hence setting the control to 1.0. The housekeeping gene GAPDH was stable throughout the experiments.

**Caspase -1 activity**

A tetrapeptide, caspase-1 enzyme substrate Ac–YVAD–AMC (Acetyl- L-tyrosyl- L-valyl- L-alanyl- L-aspartic acid a-(4- methyl- coumaryl- 7-amide) (PeptaNova GmbH, Keplerstr, Sandhausen, Germany) was used to measure the caspase-1 activity in NECs. NECs were grown in 96 well–plates and were infected with S. aureus strains (MOI 50; Cowan1, 06T203, 06T307, 07T1307) for 1 to 8h or treated with budesonide (0, 10, 100 and 1000 nM) for 24h. Caspase-1 activity was measured at 1h, 2h, 3h, 4h, 5h, 6h, 7h and 8h time points. Briefly, 5 μl caspase-1 substrate was added in each well and cells were incubated under cell culture conditions. The cleaved and released fluorogenic AMC which determines caspase-1 activity in NECs was measured using Fluostar optima (BMG Labtechnologies, Offenburg, Germany). Wells with medium only served as blank and fluorescence values were presented after blank subtraction.

**Enzyme-linked immunosorbent (ELISA) assay of IL-1β secretion by NECs**

ELISA kit (R&D systems, Stockholm, Sweden) was used to compare the levels of secretory IL-1β. Cell culture supernatants were obtained after 8h of infection with S. aureus strains (50MOI; Cowan1, 06T203, 06T307, 07T1307) and after 24h of treatment with budesonide (0, 10, 100 and 1000nM).

**Statistical analysis**

All results from infection and budesonide treatments are representative of four independent experiments. The statistical analyses performed with GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Data analyzed for significance using unpaired t-test, paired t-test or one–way analysis of variance (ANOVA) with post hoc Dunnett’s multiple comparison test depending on the nature of the data. Statistical significance was represented as p < 0.05 as *; p < 0.01 as **; and p<0.001 as ***.

**Results**

**Viability assay**

We found that after 2h, 8h and 24h exposure at 50MOI to early log phase bacteria > 90, about 70 and 35 percent cells respectively, were viable (data not shown). Therefore, for the mRNA expression analysis experiments, to get the highest yield of RNA, it was decided to infect the cells with S. aureus at 50MOI for 2h. While in other infection experiments, 8h was chosen as the endpoint.

**Visualization of intracellular and adhered S. aureus within and on (NECs)**

All the S. aureus strains used in this study, adhered and internalized the NECs (Figure 1 a–d).

**Gene expression analysis**

The results showed that the relative mRNA expression of NLR1, NLR2, IL-1β and caspase–1 measured in patient group were significantly elevated when compared to healthy controls. However, there were no statistical differences in the mRNA expression of the NLR3 between patient and control groups (Figure 2 a–e). The mRNA expression of these NLRs, caspase-1 and IL-1β was not significantly changed by S. aureus strains (data not shown). Treatment with budesonide (1000nM) decreased mRNA levels of NLR2 but did not show any significant effect on the mRNA expression of the NLR3, caspase-1 activity and IL-1β (Figure 3). We could not find any statistically significant differences in the levels of these innate immunity mediators between patients with CRSsNP and CRSwNP (data not shown).

**Caspase-1 activity in NECs**

Infection by S. aureus increased the caspase-1 activity in a time dependent manner in NECs (Figure 4). Treatment with varying concentration of budesonide did not show any significant effect on the caspase-1 activity in the NECs (data not shown).
IL-1β concentration

The minimum detectable dose of human IL-1β in the kit used in this study was determined to be 0.8 pg/ml. The levels of IL-1β in the cell culture supernatants obtained from NECs at passage 0, from CRS patients and the healthy controls remained undetectable (data not shown). Infection by S. aureus significantly increased the concentrations of IL-1β in the cell culture supernatants (Figure 5 a), while the treatment with 1000 nM budesonide decreased the concentrations of IL-1β (Figure 5 b).

Discussion

The inflammatory response is the most important component in the pathogenesis of CRS. In CRS, S. aureus may play a role in the severity of disease by activating the immune response, which may drive CRS-associated inflammation [20,26–28]. Studies have shown that NLRP3 plays a significant role in the chronic airway inflammation, including CRS [12,14,15].

In the present study, we found that in the primary NECs the mRNA levels NLRC1, NLRC2, caspase-1 and IL-1β were significantly increased in CRS group when compared to the healthy controls. The mRNA levels of the NLRP3 demonstrated a trend towards elevation in the CRS group, but the data remained non-significant. Additionally, the concentrations of IL-1β in the cell culture supernatants obtained from NECs at passage 0, remained undetectable. Infection with S. aureus increased IL-1β secretion by the NECs. Treatment with budesonide (1000 nM) decreased mRNA levels of NLRC2 and protein secretion of IL-1β but did not show any significant effect on the mRNA expression of NLRP3 and caspase-1 activity. While caspase-1 activity increased in a time dependent manner in NECs upon S. aureus infection.

Epithelial cells have innate immune functions and airway epithelial cells are involved in providing localized innate immune defenses against microbes [22,29]. In this study,
we found a higher expression of NLRC1, NLRC2 and a trend towards increased NLRP3 expression in CRS group compared to healthy controls. A similar finding was reported by Mansson et al. (2011) showing an elevated levels of NLRC1, NLRC2 and NLRP3 in the nasal epithelia of nasal polyposis patients when compared to the healthy controls [30]. The NLRs are cytoplasmic microbial receptors and therefore can detect intracellular PAMPs and DAMPs [13]. Activation of NLRC1 and NLRC2 have been associated with a number of chronic inflammatory diseases [31]. Our findings, therefore, support the notion that increased expression of these innate immune mediators may be associated with the persistent hyperinflammation in the nasal and sinus mucous membranes in CRS patients. Inflammation is the hallmark of CRS, where patients with nasal polyps show significantly higher levels of cytokineprofile, IL-1β, NLRP3 and caspase-1 when compared with patients without nasal polyps [12,32]. However, in this study the differences in the mRNA expression of NLRC1, NLRC2, NLRP3, Caspase 1 and IL-1β between CRSwNP and CRSsNP patients remained non-significant. This inconsistency may be due to that this study with small sample size lacks sufficient ability to reveal significant differences. This finding needs to be investigated by studying larger samples from patients with both types of CRS. NLRP3 is the most clinically implicated inflammasome as it is activated by a variety of stimuli [11]. The activation of the NLRP3 inflammasome in response to pathogen, leads to the activation of caspase-1, which further can lead to the activation and secretion of IL-1β [1,13,19,33]. The activation of NLRP3 inflammasome in the airway epithelia may contribute to inflammatory responses [14]. Our result showed a trend towards an increased mRNA expression of NLRP3 in the CRS group. However, the data remained non-significant. While, Lin et al (2016), reported an overexpression of NLRP3 and caspase-1 in CRS patients, especially in patients with eosinophilic CRSwNP [12]. This partial inconsistency may be due to differences in the patient group, included in this study, as in the CRS group we had recruited both types of patients with and without nasal polyps, and size of our study population was relatively small (n=14). Large sample size might have shown a clearer result. Moreover, as S. aureus infection is common in sinus mucosa, we wanted to investigate the effects of clinical isolates of S. aureus on the mRNA expression of NLRC1, NLRC2, NLRP3, caspase-1 and IL-1β. NECs, obtained from healthy controls, were infected separately with each S. aureus strains (cowan1, 06T203, 06T307 and 07T1307) at 50MOI for 8h (n=3) or treated with budesonide for 24h (n=4). Infection with S. aureus induced a significant increase in IL-1β production (a), whereas 1000nM budesonide treatment slightly decreased its production by NECs (b). A statistical significance with p<0.05 as *, p<0.01 as **, p<0.001 as *** was calculated by using paired t-tests.

The chronic inflammation in the sinus tissue is due to the presence of higher levels of cytokines in sinus secretions [18,19,35]. The levels of secretory IL-1β, in the cell culture supernatants of NECs, from CRS and control groups remained undetectable. The minimum detectable concentration of IL-1β, in the kit we used, was determined to be 0.8pg/ml. In order to avoid the effects of possible cell culture stresses on the cells, in vitro, we used cells at passage 0 for this comparison, but the levels of IL-1β remained undetectable, whereas NECs at passage 1 (used in S. aureus or budesonide exposure experiments) produced detectable IL-1β. IL-1β is important for acute inflammatory responses to infection, but excessive secretion contributes to hyperinflammatory conditions and tissue damage. Inhibition of IL-1β by anti-inflammatory drugs like corticosteroids is beneficial. We found that at high concentration (1000nM) budesonide treatment slightly decreased IL-1β secretion and the effect of varying concentrations of budesonide on the caspase-1 activity remained non-significant. However, this study has some limitations. The S. aureus induced increase in caspase-1 activity and IL-1β secretion in NECs were not studied in the presence of budesonide. So, it is needed to confirm the effectiveness of budesonide in the presence of S. aureus infection. Moreover, at transcriptional levels, only NLRC2 expression was down regulated by 1000nM budesonide, which indicated that these data were partly consistent with the published reports [12,30,36]. Lennard et al. (2000) has also reported that prednisone (corticosteroids) reduces the increased levels of proinflammatory cytokines in the sinonosal mucosa of CRS patients [19]. On the other hand, there are indications suggesting that corticosteroids have little or no inhibitory effect on innate immunity in the airway epithelia [22]. Furthermore, an opposing effect of budesonide has been shown, in reducing IL-6 and IL-8 production and enhancing expression of TLR2 in primary bronchial epithelial cells [37].
However, our result showed that budesonide exert inhibitory effects, at least on the NLRC2 and IL-1β secretion in the NECs.

**Conclusion**

Taken together, our results suggest that activation of caspase-1 in the NECs by *S. aureus* may have an important role in the pathogenesis of CRS by inducing increased secretion of IL-1β. Budesonide exerts its anti-inflammatory effect in the NECs by attenuating the release of IL-1β.

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