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Published in: Respiratory Research

DOI: 10.1186/1465-9921-13-2

2012

Link to publication

Citation for published version (APA):
Kvarnhammar, A. M., Rydberg, C., Jarnkrants, M., Eriksson, M., Uddman, R., Benson, M., & Cardell, L-O. (2012). Diminished levels of nasal S100A7 (psoriasin) in seasonal allergic rhinitis: an effect mediated by Th2 cytokines. Respiratory Research, 13. https://doi.org/10.1186/1465-9921-13-2

Total number of authors: 7

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Diminished levels of nasal S100A7 (psoriasin) in seasonal allergic rhinitis: an effect mediated by Th2 cytokines

Anne Månsson Kvarnhammar1*, Camilla Rydberg1, Malin Järnkrants1, Mia Eriksson1, Rolf Uddman2, Mikael Benson3 and Lars-Olaf Cardell1

Abstract

Background: S100A7 is an antimicrobial peptide involved in several inflammatory diseases. The aim of the present study was to explore the expression and regulation of S100A7 in seasonal allergic rhinitis (SAR).

Methods: Nasal lavage (NAL) fluid was obtained from healthy controls before and after lipopolysaccharide (LPS) provocation, from SAR patients before and after allergen challenge, and from SAR patients having completed allergen-specific immunotherapy (ASIT). Nasal biopsies, nasal epithelial cells and blood were acquired from healthy donors. The airway epithelial cell line FaDu was used for in vitro experiments. Real-time RT-PCR and immunohistochemistry were used to determine S100A7 expression in nasal tissue and cells. Release of S100A7 in NAL and culture supernatants was measured by ELISA. The function of recombinant S100A7 was explored in epithelial cells, neutrophils and peripheral blood mononuclear cells (PBMC).

Results: Nasal administration of LPS induced S100A7 release in healthy non-allergic subjects. The level of S100A7 was lower in NAL from SAR patients than from healthy controls, and it was further reduced in the SAR group 6 h post allergen provocation. In contrast, ASIT patients displayed higher levels after completed treatment. S100A7 was expressed in the nasal epithelium and in glands, and it was secreted by cultured epithelial cells. Stimulation with IL-4 and histamine repressed the epithelial S100A7 release. Further, recombinant S100A7 induced activation of neutrophils and PBMC.

Conclusions: The present study shows an epithelial expression and excretion of S100A7 in the nose after microbial stimulation. The levels are diminished in rhinitis patients and in the presence of an allergic cytokine milieu, suggesting that the antimicrobial defense is compromised in patients with SAR.

Keywords: allergen-specific immunotherapy, antimicrobial peptide, epithelium, lipopolysaccharide, seasonal allergic rhinitis, Th2 cytokines

Background

S100A7, also known as psoriasin, belongs to the S100 protein family consisting of ~20 different EF-hand type proteins [1]. It was first identified as highly up-regulated in the skin of psoriatic patients [2], but has also been attributed a role in atopic dermatitis (AD) and different types of cancer, including skin, breast and bladder cancer [3–6]. The function of S100A7 is still poorly understood, but several studies suggest that it acts both as an antimicrobial peptide (AMP) and as a chemotactic factor for neutrophils and CD4+ T cells [7–9]. In addition, S100A7 has been shown to directly kill bacteria and protect human skin from E. coli infection [7], and to activate neutrophils to produce a range of cytokines, chemokines, AMPs and reactive oxygen species [10].

Although S100A7 has been linked to atopic dermatitis, there is little information available regarding its role in other atopic diseases. We have previously shown lower levels of S100A7 in nasal lavage (NAL) fluid from patients with pollen-induced seasonal allergic rhinitis...
SAR patients

Lipopolysaccharide (LPS) and allergen provocation

Sample collection

Airway epithelial cells (AEC)

Subjects

Methods

Results

Discussion

Table 1 Patient characteristics.
0.4% bovine pituitary extract, 10 ng/ml epidermal growth factor, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.5 μg/ml epinephrine, 6.7 ng/ml triiodothyronine, 10 μg/ml transferrin, 0.1 ng/ml retinoic acid (PromoCell, Heidelberg, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) [20]. All cells were cultured at 37°C in a humidified 5% CO2 air atmosphere.

FaDu cells were seeded on 24-well culture plates (250,000 cells/well) in 1 ml complete MEM and incubated overnight. Thereafter, they were cultured for additionally 24 h in the absence or presence of recombinant human IL-4 (R&D Systems, Minneapolis, MN, USA), histamine (Sigma-Aldrich) or S100A7 (ProtEra, Sesto Fiorentino, Italy).

**Isolation and culture of cells from peripheral blood**

Peripheral blood was acquired from healthy volunteers, diluted 1:1 in PBS and centrifuged using Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden). Peripheral blood mononuclear cells (PBMC) were retrieved from the interphase fraction, whereas granulocytes were recovered from the pellet. Briefly, the granulocyte-rich pellet, containing ≥ 90% neutrophils, was treated with an ammonium chloride lysing buffer to remove erythrocytes, followed by collection of neutrophils. Eosinophils (Sigma-Aldrich) or S100A7 (ProtEra, Sesto Fiorentino, Italy).

Peripheral blood was acquired from healthy volunteers, diluted 1:1 in PBS and centrifuged using Ficoll-Paque (Amersham Bioscience, Uppsala, Sweden). Peripheral blood mononuclear cells (PBMC) were retrieved from the interphase fraction, whereas granulocytes were recovered from the pellet. Briefly, the granulocyte-rich pellet, containing ≥ 90% neutrophils, was treated with an ammonium chloride lysing buffer to remove erythrocytes, followed by collection of neutrophils. Eosinophils were negatively selected from granulocytes using the MACS magnetic labeling system (Eosinophil Isolation Kit, Miltenyi Biotec, Cologne, Germany) as previously described in detail [21]. The interphase fraction after centrifugation was washed, and monocytes were isolated using CD14 Microbeads (Miltenyi Biotec). B and T lymphocytes were negatively selected using the B cell Isolation Kit II and Pan T cell Isolation Kit II [22,23], respectively (Miltenyi Biotec).

PBMC (1 × 10^6 cells/ml) and neutrophils (4 × 10^6 cells/ml) were cultured in RPMI 1640 supplemented with 0.3 g/l L-glutamine (PAA, Pasching, Austria), 10% FBS, 100 U/ml streptomycin and 100 μg/ml penicillin in the absence or presence of rhS100A7. After various time points, cytokine levels in the supernatants were analyzed using ELISA, proliferation was measured by [methyl-3H] thymidine incorporation and expression of the adhesion molecule CD11b was determined using flow cytometry.

**Immunohistochemistry**

The presence of S100A7 protein in the nose was assessed using immunohistochemical staining. Nasal biopsies were fixed in 4% buffered formaldehyde for 7 days, thereafter processed and embedded in paraffin. Sections (3 μm thick) were cut, dried for 2 h at 60°C and then stored at 4°C until further use. The tissue sections were deparaffinized in xylene and then rehydrated through graded alcohol and deionized water. For antigen retrieval the sections were processed in a microwave oven in target retrieval solution with a pH of 6.1 (Dako, Glostrup, Denmark). A mouse anti-human S100A7 antibody (clone 47C1068) from Abcam (Cambridge, UK) was used at a dilution of 1:100. As negative control, Negative Control Reagent against mouse (Dako) was utilized. The staining procedure was performed in a Techmate 500 Plus immunostaining machine according to standard "ENVP" procedure (Dako). The sections were counterstained with hematoxylin, dehydrated in graded alcohol, passed through xylene and mounted.

**RNA extraction and real-time RT-PCR**

Nasal biopsies, epithelial cells and freshly isolated leucocytes were lysed in RLT buffer (Qiagen, Hilden, Germany) supplemented with 1% 2-mercaptoethanol and stored in -80°C until use. 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 primer (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.

The real-time PCR reactions were performed on a Stratagene Mx3000P (Agilent Technologies, Santa Clara, CA, USA) using the Brilliant® II SYBR® Green QPCR Mastermix (Agilent Technologies) in a final volume of 20 μl. The PCR primers (β-actin fwd: 5′-GCCAACCGCGAGACTGATGGT-3′, rev: 5′-ACGCGCAGGCTTCCAGACAG-3′; S100A7 fwd: CGTGAAGGTCTCCACTCT-3′, rev: 5′-TCATACCGTCTTGGATCCA-3′) were designed using Primer Express® 2.0 Software (Applied Biosystems, Foster City, CA, USA) and synthesized by DNA Technology [12]. The thermal cycler was set to perform 95°C for 15 min, followed by 46 cycles of 94°C for 30 s and 55°C for 60 s (initially 65°C, followed by a 2°C decrease for the six first cycles). Melting curve analysis was performed to ensure specificity of the amplified PCR products. 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 S100A7 with Ct values of β-actin (ΔCt). The amount of mRNA is expressed as 2^-ΔCt × 10^5 [19-23].

**Flow cytometry**

Cultured neutrophils were stained with CD16-ECD (clone 3G8) and CD11b-FITC (Becton) (Beckman Coulter, Marseille, France) for 15 min in RT. Thereafter, cells were washed, resuspended in PBS and analyzed on a Coulter Epics XL flow cytometer (Beckman Coulter). Neutrophils were distinguished based on their forward
scatter (FSc) and side scatter (SSc) properties as well as their expression of CD16, as FSc$^{\text{high}}$, SSc$^{\text{high}}$, CD16$^+$. 10,000-15,000 events were collected and analyzed using Expo32 ADC analysis software (Beckman Coulter).

**ELISA**

Levels of S100A7 in unprocessed NAL fluid, serum and culture supernatants were detected using the Circulex™ S100A7/Psoriasin ELISA kit (detection limit 0.12 ng/ml) from MBL International (Woburn, MA, USA). ELISA plates to measure IL-1β (1 pg/ml), IL-6 (0.7 pg/ml), IL-8 (3.5 pg/ml) and IL-10 (3.9 pg/ml) in cell culture supernatants were obtained from R&D Systems.

**Statistics**

Statistical analyses were performed using GraphPad Prism 5 (San Diego, CA, USA). All data are presented as mean ± standard error of the mean (SEM), and $n$ equals the number of independent donors. For unpaired data, statistical differences were determined using unpaired Student’s $t$-test with Welch correction if the variances were non-homogenous. Paired observations were analyzed using paired $t$-test (for two sets of data) or one-way repeated measures ANOVA with Dunnett’s post test (> 2 sets of data). A $p$-value of < 0.05 was considered statistically significant.

**Results**

**Nasal LPS administration induces release of S100A7**

To investigate whether microbial exposure affects the nasal release of S100A7, healthy non-allergic subjects were exposed to nasal administration of the prototypical inflammatory stimulus LPS. NAL fluids were collected before and 24 h post challenge followed by measurements of S100A7 by ELISA. LPS was found to increase the S100A7 excretion (Figure 1).

**Levels of S100A7 in NAL fluid are lower in patients with SAR and decrease after allergen provocation**

NAL fluids and serum from healthy subjects and SAR patients outside season were analyzed for levels of S100A7 by use of ELISA. A significantly lower baseline S100A7 release in the nose was seen in the allergic as compared to the healthy group (Figure 2A), whereas no difference was found in serum (4.5 ± 0.9 vs. 3.7 ± 0.5 ng/ml; $p = 0.74$). Thereafter, we wanted to investigate the effects of allergen provocation on nasal S100A7 secretion in the allergic group. It was found that the S100A7 levels were significantly lower 6 h post provocation as compared to before (Figure 2B).

**Levels of S100A7 increase in SAR patients after ASIT**

The diminished levels among allergics led us to investigate whether SAR patients that have completed ASIT treatment still have a deficient S100A7 production or whether they have acquired levels comparable to those of healthy individuals. NAL fluid was collected from 10 patients before and after ASIT and analyzed for baseline levels of S100A7 by use of ELISA. Immunotherapy treatment increased the generation of S100A7 in nine out of the ten donors examined (Figure 2C).

**S100A7 is produced by nasal epithelial cells**

To ascertain the presence of S100A7 in the nose, immunohistochemistry and real-time RT-PCR were performed on nasal biopsies obtained from healthy donors. An intense immunostaining was seen in seromucous glands and in the epithelium (Figure 3A, B), whereas replacement of the primary specific antibody with an isotype-matched control antibody resulted in complete loss of staining (Figure 3C). Expression of S100A7 mRNA was found in all biopsy specimens (Figure 3D).

The cellular sources of S100A7 were further investigated in primary HNEC, the nasopharyngeal epithelial cell line FaDu and isolated leukocytes that can be found in the epithelial and subepithelial regions. A distinct mRNA expression was seen in HNEC, but also in FaDu (Figure 3D), and secreted S100A7 could be detected in the cell culture medium from both AECs (Figure 3E). Low levels of S100A7 mRNA were also found in B cells, monocytes and neutrophils, whereas there was no expression in T cells or eosinophils (Figure 3F).
Figure 2 Diminished levels of nasal S100A7 in allergic patients (A) Baseline levels of S100A7 were measured by ELISA in nasal lavage (NAL) fluids obtained from healthy subjects (n = 13) and patients with seasonal allergic rhinitis (SAR, n = 12). **, p < 0.01 (unpaired t-test). (B) Levels of S100A7 in NAL fluid from patients with SAR before and 6 h after allergen provocation (n = 12). ***, p < 0.001 (paired t-test). (C) Baseline levels of S100A7 in NAL fluid obtained from patients with SAR before and 3 years after allergen-specific immunotherapy (ASIT, n = 10). *, p < 0.05 (paired t-test).

Figure 3 S100A7 is present in the nasal epithelium and seromucous glands. Tissue slides of the human inferior turbinate were stained with (A, B) a monoclonal antibody against S100A7 or (C) an isotype-matched control antibody. Positive staining (brown) was detected in the nasal epithelium and in seromucous glands. Counterstaining with hematoxylin (blue) (magnification 100 ×). (D) Expression of S100A7 mRNA was determined in nasal biopsies (n = 11), primary human nasal epithelial cells (HNEC, n = 6) and FaDu (n = 4) by real-time RT-PCR. Data is expressed in relation to β-actin as $2^{-\Delta\Delta C_T} \times 10^5$. (E) Levels of S100A7 in the culture medium from HNEC and FaDu were analyzed by ELISA (n = 4-6). (F) mRNA expression of S100A7 in purified peripheral blood T lymphocytes, B lymphocytes, monocytes, neutrophils and eosinophils using real-time RT-PCR (n = 3-6). Data are presented as mean ± SEM.
Th2 cytokines repress the S100A7 secretion by epithelial cells

To study the regulation of S100A7 in the nose and elucidate the mechanisms behind the diminished levels during SAR, we hypothesized that it might be consumed during the allergic inflammation by the infiltrating cells. The target cells for secreted S100A7 in the nose are not fully identified, but the reported chemotactic properties for CD4+ T cells and neutrophils [8] suggest that it is aimed for the latter as neutrophils are the most abundant cell type in nasal washings [24].

The number of neutrophils in NAL fluid was significantly higher in the allergic as compared to the healthy group (6.4 ± 1.8 vs. 1.6 ± 0.7 × 10^6 cells/ml; p < 0.05), whereas allergen provocation did not alter the number of cells (5.7 ± 0.8 vs. 6.4 ± 1.8 × 10^6 cells/ml; p = 0.70). However, no correlation between neutrophil numbers and S100A7 levels was found (r^2 = 0.055; Pearson r = -0.235; p = 0.21).

Instead, we asked whether mediators produced during the allergic inflammation per se could have a role in the regulation of S100A7. FaDu was cultured for 24 h in the absence or presence of IL-4 and histamine followed by measurements of S100A7 levels. Both mediators markedly repressed the epithelial S100A7 release (Figure 4).

S100A7 activates PBMC and neutrophils

The last set of experiments aimed to explore the ability of S100A7 to activate different cellular subsets. The epithelial cell line FaDu, PBMC and neutrophils were stimulated with rhS100A7 for various time points followed by measurements of cytokine secretion, proliferation and adhesion molecule expression. In epithelial cells, S100A7 promoted neither IL-6 nor IL-8 secretion after 24 h of stimulation (data not shown). PBMC, on the other hand, were induced to release IL-6, IL-8 and IL-10, as well as proliferate in response to S100A7 (Figure 5A-D). Likewise, neutrophils were activated to produce IL-1β, IL-6 and IL-8, and up-regulate the expression of the adhesion molecule CD11b (Figure 5E-H).

Discussion

The present study describes S100A7 in the nose and how it is regulated during SAR. Nasal administration of LPS induces S100A7 release in healthy non-allergic subjects. Lower levels of S100A7 are seen in NAL fluid from SAR patients than from healthy controls and the levels are further decreased upon allergen provocation. SAR patients having completed ASIT display higher levels of S100A7 in NAL than before initiation of treatment. S100A7 is found to emanate mainly from the nasal epithelium and to activate PBMC and neutrophils to produce cytokines, proliferate and up-regulate adhesion molecule expression. Further, the epithelium-induced S100A7 generation is repressed by the allergic mediators IL-4 and histamine.

We have previously identified S100A7 in the NAL proteome using 2-dimensional gel electrophoresis in combination with mass spectrometry, and demonstrated lower levels in patients with symptomatic SAR compared to controls [11]. The present study, investigating S100A7 in NAL using ELISA, confirms and complements previous data by showing diminished levels in SAR patients outside pollen season compared to non-allergic controls, along with a further reduction in the SAR group after allergen provocation. Moreover, the levels appear to return to normal after immunotherapy treatment. No differences in S100A7 levels among healthy and allergic subjects are seen in serum, which supports the notion of a local role for S100A7 in SAR. Results from immunohistochemical staining and real-time RT-PCR of nasal biopsies clearly demonstrate presence of S100A7 in the epithelium and seromucous glands, and culture of epithelial cells reveals a basal secretion of S100A7. These findings are in accordance with a study by Glaser et al. showing an expression in sebaceous glands in the nose [7], and recent results by Tieu and colleagues demonstrating reduced levels of epithelial S100A7 in patients with CRS and SAR [14,15]. It should also be mentioned that in contrast to the low

![Figure 4](http://respiratory-research.com/content/13/1/2)
levels of S100A7 seen during inflammatory conditions in the upper airways, there are studies demonstrating an enhanced expression and secretion of S100A7 in the skin of patients with AD [25-27]. Moreover, we have previously detected S100A7 in tonsillar epithelium and lymphocytes (CD19+, CD4+ and CD8+ cells) and found reduced levels in tonsils in response to infection and atopic predisposition [12]. In contrast, blood-derived B and T cells express very low or no levels of S100A7. These discrepancies in expression might be related to compartmental differences. Also, the high antigen load in the tonsils might cause a microbe-induced regulation of S100A7 expression.

Accumulating evidence suggests that there might be intrinsic or disease-driven deficiencies in the epithelial barrier function of the nasal mucosa in AR patients [28]. Indeed, allergies suffer from an ongoing so called minimal persistent inflammation, characterized by e.g. infiltration of inflammatory cells (eosinophils and neutrophils) and up-regulation of epithelial adhesion molecules, which in turn primes the nasal mucosa leading to increased sensitivity and responsiveness to allergen provocation [29,30]. Likewise, we have recently shown that SAR patients have higher clinical symptom scores, pulmonary nitric oxide production, NAL leukocyte numbers and cytokine levels compared to healthy subjects [16]. The epithelium provides not only a physical barrier through mucociliary clearance and tight junctions, but it can also resist entry of pathogens through the production of antimicrobial defense proteins. The present study shows reduced levels of S100A7 in SAR patients and in response to allergen challenge. In addition to the epithelial disruption seen in the atopic airway [31], we propose defects in AMPs of this kind to be a factor behind the increased susceptibility to microbial infection often seen during periods of SAR [32-34]. However, patients having completed ASIT have a less pronounced minimal persistent inflammation, manifested by a reduced eosinophilic infiltration into the nasal mucosa and reduced epithelial damage [35], which in turn can explain the higher levels of S100A7 in NAL fluid among these patients.

Nasal challenge with LPS, as a mimic of bacterial upper airway infection, induces release of S100A7 in NAL fluid from healthy subjects. In line with this, the expression of S100A7 by the human hair follicle epithelium has been found to be inducible upon treatment with prototypical microbial products such as LPS [36]. Interestingly, unpublished data from our lab show that nasal administration of LPS (total dose of 50 μg) in SAR

**Figure 5** S100A7 activates PBMC and neutrophils (A-D) PBMC were cultured (1 × 10^6 cells/ml) for 48 and 72 h with or without S100A7. Thereafter, cell culture supernatants were analyzed for levels of IL-6, IL-8 and IL-10 by use of ELISA, and the proliferative capacity of the cells was determined by [methyl-^-3H]thymidine incorporation (n = 6). (E-H) Neutrophils were cultured (4 × 10^6 cells/ml) for 4 and 18 h in the absence or presence of S100A7. Levels of IL-6, IL-8 and IL-1β were measured in the supernatants by ELISA, and expression of CD11b was analyzed by flow cytometry (n = 6). Data are expressed as mean ± SEM. *, p < 0.05; **, p < 0.01; ***, p < 0.001 (ANOVA).
patients does not induce release of S100A7 in NAL (before: 92.2 ± 6.5 vs. 24 h after: 92.0 ± 7.4, p = 0.98). The inability of these patients to respond in terms of S100A7 secretion might be a consequence of their impaired antimicrobial defense system. However, it cannot be excluded that the differential responsiveness to LPS among healthy and allergic subjects is related to the dose, as a 2-fold lower concentration was given to the latter group.

The diminished levels of S100A7 during SAR do not correlate with the infiltration of neutrophils. Instead, IL-4 and histamine are found to suppress the S100A7 secretion by epithelial cells, suggesting that a Th2-like cytokine milieu negatively regulates the S100A7 production. Elevated levels of Th2-biased cytokines have been demonstrated in nasal washings of SAR patients both during pollen season and after allergen provocation [37-39], and Glaser et al. have demonstrated that the TNF-α-induced S100A7 secretion by human keratinocytes is inhibited by IL-4 and IL-13 [25].

Even though there is a ~2-fold reduction in the concentration of S100A7 in NAL fluid from SAR patients, it should be mentioned that the changes in levels in some of the treatment groups are fairly small, which raises questions regarding the biological significance. However, in real-life, efficient clearance of microbes involves a crosstalk between several different cells, signals and mediators that are triggered simultaneously. This is reflected by e.g. the ability of S100A7 to activate the cellular immunity and the ability of cytokines, including TNF-α, IL-4, IL-6 and oncostatin-M [3, 25], to both positively and negatively regulate the generation of S100A7. Hence, it is not the effect of each single AMP, but the combined actions of different mediators that are of biological importance.

The receptor for secreted S100A7 is still unknown, and consequently its functional relevance is poorly understood. In the present study, we demonstrate that S100A7 activates human PBMC and neutrophils to produce cytokines, proliferate and up-regulate adhesion molecule expression. Neutrophils have previously been reported to secrete cytokines, reactive oxygen species and AMPs in response to S100A7 stimulation [10], but so far there are no other studies demonstrating an effect on mononuclear cells except for its chemotactic properties for CD4+ T cells [8].

**Conclusion**

Present data propose that the nasal epithelium produces S100A7 in response to microbial stimulation, which in turn attracts immunocompetent cells in order to mount a proper antimicrobial defense (Figure 6). However, during an allergic inflammation, the Th2-type cytokine response represses the S100A7 release. As a result of the diminished S100A7 levels, allergic patients have reduced bactericidal properties as well as a decreased capability to activate the cellular immunity, which in turn might render them more susceptible to microbial colonization and/or make their conditions worse once an infection is established. In line with this, it is tempting to speculate that ASIT-treated patients, who have regained a normal S100A7 production, in turn have a reduced propensity to develop upper respiratory tract infections compared to their untreated counterparts.

**List of abbreviations**

AEC: airway epithelial cells; AD: atopic dermatitis; AMP: antimicrobial peptide; ASIT: allergen-specific immunotherapy; CRS: chronic rhinosinusitis; Ct: cycle threshold; HNEC: human nasal epithelial cells; LPS: lipopolysaccharide; NAL: nasal lavage; PBMC: peripheral blood mononuclear cells; rh: recombinant human; SAR: seasonal allergic rhinitis; SPT: skin prick test

**Acknowledgements**

This study was supported by the Swedish Medical Research Council, the Swedish Heart-Lung Foundation, Karolinska Institutet and the Karolinska University Hospital. The authors wish to thank Ingegerd Larsson for skillful technical assistance during this study.

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**Authors’ contributions**

AMK performed most of the experiments, analyzed the data and wrote the paper. CR carried out the experiments with the primary epithelial cells. MJ...
performed the nasal biopsy experiments, ME the neutrophil and PBMC cultures and RU the immunohistochemical analyses. MB helped with the design of the study and revised the manuscript. LOC provided financial support and critically revised the manuscript. All authors have read and approved the final form of the manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 3 October 2011 Accepted: 9 January 2012
Published: 9 January 2012

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