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Increased expression of IL-19 in the epithelium of patients with chronic rhinosinusitis and nasal polyps

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Abstract

Background: Chronic rhinosinusitis (CRS) is an inflammation of the nose and of the paranasal sinuses. The involvement of the respiratory epithelium in the mechanisms of CRS is poorly understood.
Aims: Among proteins expressed by nasal epithelial cells in CRS, IL-19 may have key functions. We here aimed to determine the expression and regulation of IL-19.
Methods: Nasal biopsies from normal subjects (n = 12), subjects with CRS but without nasal polyps (NP) (CRSsNP, n = 12) and with CRS with NP (CRSwNP, n = 15) were collected. Human Asthma Gene Array and real-time PCR were used to evaluate gene expression, western blot analysis and immunohistochemistry for protein expression. Results for IL-19 were confirmed by real-time PCR. The constitutive and stimulated (LPS, TGF β) expression of IL-19 and cell proliferation were evaluated in a nasal epithelial cell line (RPMI 2650).
Results: Human Asthma Gene Array showed an increased IL-19 gene expression in NP from patients with CRS in comparison with normal subjects. Real-time PCR confirmed the IL-19 mRNA up-regulation in patients with CRSwNP and showed an up-regulation of IL-19, at lower extent, in patients with chronic rhinosinusitis without nasal polyps (CRSsNP) in comparison with normal subjects. Western blot analysis confirmed that IL-19 is increased also at protein level in patients with CRSwNP in comparison with normal subjects. In NP, IL-19 is highly expressed in the metaplastic nasal epithelium when compared to normal or hyperplastic epithelium. LPS stimulation increased IL-19 expression, and recombinant IL-19 increased cell proliferation in nasal epithelial cells.
Conclusions: IL-19 is overexpressed in the epithelium in CRSwNP and increases epithelial cell proliferation.

Chronic rhinosinusitis (CRS) is an inflammation of the nose and of the paranasal sinuses, which may be differentiated in CRS without (CRSsNP) and with nasal polyps (CRSwNP) based on remodelling and inflammatory patterns (1, 2). CRSwNP is mainly characterized by eosinophilic inflammation and prevalent helper T cell type(Th)-2 responses while CRSsNP is characterized by neutrophilic inflammation and prevalent Th1 responses (3). The aetiology and pathogenesis of CRS are largely unknown, and abnormalities in host response to external insults including allergens, fungi, bacteria and virus have been suggested to underlie the persistence of the inflammatory state (4). Among these, *Staphylococcus aureus* may play an important role by releasing enterotoxins, acting as superantigens and amplifying the Th2-biased

Abbreviations
CRS chronic rhinosinusitis; CRSwNP chronic rhinosinusitis with nasal polyps; CRSsNP chronic rhinosinusitis without nasal polyps; IL interleukin; NP nasal polyps.

immune response (5). Histological features of CRSwNP resemble that of asthmatic airways with end-stage polyps displaying signs of Th2 inflammation characterized by infiltration with eosinophils, thickening of the basement membrane, and hyperplasia of the epithelium and are strikingly reminiscent of the histopathology of severe asthmatic airways (6). Patients with severe and/or uncontrolled asthma have more severe nasal computed tomographic (CT) scan abnormalities than other patients (7). Multiple mechanisms may contribute to the increase in Th2 responses. In asthmatic patients, IL-17A and IL-4/IL-13 synergistically up-regulate IL-19 expression in airway epithelium, which in turn induces the expression of Th2 cytokines in activated T lymphocytes (8). Nasal epithelium plays a crucial role in innate and adaptative immune responses, and alterations in epithelial barrier function and host defence responses may contribute to CRS pathogenesis (9,10). The immune mechanisms promoting the structural alterations in CRS are not well elucidated.

In the present study, based on a gene array, we focus on IL-19 RNA and protein expression and regulation of its production as a possible key cytokine in the regulation of Th2 responses in CRS with NP.

Materials and methods

Study population

Patients were recruited at the ENT departments of Palermo, Italy, and of Ghent, Belgium. We selected subjects without CRS (n = 12) (age, 18–48 year) and without allergic diseases or asthma (controls), subjects with CRS but without NP (CRSsNP) (n = 12) (age, 23–51 year) and subjects with CRS and NP (CRSwNP) (n = 15) (age, 20–48 year) (11). The study fulfilled the criteria of the Ethics Committee of both hospitals and was in agreement with the Helsinki Declaration. All subjects had given their written informed consent. Further details are provided in the Supporting information.

Nasal biopsies

Nasal biopsies were collected from inferior turbinates or from nasal polyps. For mRNA analyses, nasal biopsies were immediately kept in RNA-latter and snap-frozen at −80°C. For immunohistochemistry, nasal biopsies were fixed (10% neutral buffer formalin) and embedded in paraffin wax. Some nasal biopsies from CRSwNP were freshly processed to isolate nasal epithelial cells as previously described (12).

cDNA expression array

Total RNA was isolated from the samples using TriZol reagent (Invitrogen, Carlsbad, CA, USA) (13), and RNA purification was performed with RNeasy Mini kit (Qiagen Inc, Valencia, CA, USA). Human Asthma Gene Array, analysing 85 asthma related genes simultaneously (SuperArray Inc., Bethesda, MD, USA), was used to compare the gene expression profiles of the collected samples as previously described (14). The averages of two GAPDH or four cyclophilin A spots were used as positive controls and set as baseline values with which the signal intensity of other spots was compared. Using these normalized data, we compared the signal intensity from the membranes using the GEarray analyzer program (SuperArray Corp., http://www.superarray.com).

Real-time quantitative RT-PCR of IL-19

Real-time quantitative PCR of IL-19 transcript was carried out on ABI PRISM 7900 HT Sequence Detection Systems (Applied Biosystems, Foster City, CA, USA) using specific FAM-labelled probe and primers (Assays on Demand; Applied Biosystems). Relative quantitation of mRNA levels was carried out with comparative C_T method ($2^{-\Delta\Delta Ct}$) (15).

Western blot analysis

The expression of IL-19 was evaluated by western blot analysis as previously described (16) using a goat polyclonal antibody anti-IL-19 (1 : 100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then stripped and incubated with goat polyclonal anti-ß-actin (Sigma-Aldrich, St Louis, MO, USA). Revelation was performed with an enhanced chemiluminescence system (GE Healthcare, Chalfont St. Giles, UK) followed by autoradiography. Negative controls were performed without primary antibody or including an isotype control antibody. Data are expressed as densitometric arbitrary units by correction with the density of the bands obtained for beta-actin.

Immunohistochemistry

Immunohistochemistry was performed using goat anti-human IL-19 polyclonal antibody (Santa Cruz Biotechnology) (1 : 50) or using mouse anti-human Ki67 monoclonal antibody (Clone MIB-1; Code n. M7240; Dako, Glostrup, Denmark) (1 : 75). For IL-19 expression, the intensity and percentage scores were multiplied to give a composite score of 1–9 for each specimen as previously described (17) (further details in the Supporting information). For the Ki67 expression, the percentage of Ki67-positive cells was assessed in normal/hyperplastic and in metaplastic epithelium of nasal polyps from CRSwNP subjects.

Immunofluorescence microscopy

Immunofluorescence staining was performed on tissue sections from CRSwNP subjects to evaluate the co-localization of IL-19 and Ki67. Immunostainings were performed using goat anti-human IL-19 polyclonal antibody (Santa Cruz Biotechnology) (1 : 50) and mouse anti-human monoclonal ki67 antibody (Clone MIB-1; Code n. M7240; Dako) (1 : 75). Double labelling was performed using a secondary fluorescein isothiocyanate-conjugated donkey anti-goat antibody and phycoerythrin (PE)-conjugated goat anti-mouse antibody (Sigma-Aldrich) in the same section. Slides were analysed by a fluorescence microscope (Axioscop 2; Zeiss, Heidelberg, Germany) at 488 lambda for the green fluorescence and at 589–610 lambda for the red fluorescence.

Nasal epithelial cell line

RPMI 2650, a nasal epithelial cell line was cultured with endotoxin (LPS 1 and 10 μg/ml–24 h) (Sigma-Aldrich) for assessing IL-19 expression and with recombinant IL-19 (r IL-19) (200 ng/ml) (R&D Systems, Minneapolis, MN, USA), herbimycin, a tyrosine kinase inhibitor (Sigma) (2 μM) and Na orthovanadate, a tyrosine phosphatase inhibitor (Sigma) (10 μM) (15), for 72 h for assessing cell proliferation as previously described (18). The time point was selected on the basis of preliminary experiments (data not shown). At the end of stimulation, cells were collected for assessing IL-19 expression and/or for cell proliferation.

Expression of IL-19 and of ki67 in nasal epithelial cells

The IL-19 protein expression was assessed by western blot (as mentioned previously) and by flow cytometry analysis. The ki67 was assessed by flow cytometry analysis. RPMI 2650 cells and primary epithelial cells isolated from a CRSwNP were used for these experiments. The expression of IL-19 and of ki67 by flow cytometry was assessed in permeabilized cells. Data are expressed as percentage of positive cells.

Cell proliferation

RPMI 2650 was stimulated with r IL-19 with/without herbimycin and Na orthovanadate. Cell proliferation was assessed using carboxyfluorescein succinimidyl ester (19) labelling assay and by clonogenic assay (15). Further details are provided in the Supporting information.

Statistics

Data are expressed as median (25–75 percentiles). Kruskal–Wallis test was performed for comparisons between patient groups. A nonparametric Mann–Whitney test was applied as the initial Kruskal–Wallis test was significant. The Spearman test was used for correlations. Student's paired t-test was used for comparisons in RPMI cell line. $P < 0.05$ was accepted as statistically significant.

Results

Demographic characteristics of the subjects

The demographic characteristics and the clinical evaluations of the studied patients are shown in Table 1.

Table 1 Demographic characteristics of the subjects

	Controls = 12	CRSsNP = 12	CRSw NP = 15
Gender (M/F)	5/7	7/5	7/8
Asthma	0/12	4/12	8/15
Atopy	0/12	5/12	7/15

CRSsNP, chronic rhinosinusitis without nasal polyps.

Increased expression of IL-19 mRNA in CRS with nasal polyps by Human Asthma Gene Array

Human Asthma Gene Array, analysing 85 asthma related genes simultaneously (Table S1 in the Supporting information), showed an greater than fivefold increase in IL-19 in CRSwNP in comparison with controls (Fig. 1A). No significant differences were observed between CRSsNP and normal controls (Fig. 1B,C).

Real-time PCR confirms the increased expression of IL-19 mRNA in CRS with nasal polyps

Real-time PCR confirmed that IL-19 mRNA was strongly up-regulated in patients with CRSwNP and showed a significant up-regulation of IL-19 mRNA, at lower extent, also in the mucosa from inferior turbinate of patients with CRSsNP in comparison with controls (Fig. 1D).

Increased expression of IL-19 protein in CRS with nasal polyps.

Western blot analysis confirmed that IL-19 is increased also at protein level in patients with CRSwNP in comparison with controls (Fig. 2A,B). There was no significant trend in the increase in IL-19 protein in CRSsNP.

Squamous metaplastic nasal epithelium shows higher expression of IL-19 protein in CRS with nasal polyps

Immunohistochemistry, applied to identify which cells express IL-19, showed that IL-19 is highly expressed in the epithelium of CRSsNP and of CRSwNP in comparison with controls (Fig. 3A). In the subepithelial compartment, mucous glands mainly expressed IL-19. In CRSwNP, higher expression of IL-19 was observed in squamous metaplastic nasal epithelium (score, 4–9) when compared to not metaplastic nasal epithelium (normal or hyperplastic epithelium) (score, 1–3) (Fig. 3B). Confirming what previously was reported (10), basal hyperplasia and mucous cell hyperplasia were observed in CRSwNP (see arrow and arrowhead in Fig. 3B). In patients with CRSwNP, IL-19 expression was independent of atopy as defined by a positive SPT (data not shown) or of asthma.

Nasal epithelial cells constitutively express IL-19

The expression of IL-19 in nasal epithelium may be the result of a paracrine or an autocrine mechanism. Nasal epithelial cells constitutively express IL-19, suggesting that the presence of IL-19 in the epithelium of the recruited subjects may represent also the effect of an autocrine production (Fig. 4). LPS (1 and 10 μg/ml) increased the percentage of IL-19-positive cells (Fig. 4A) and the constitutive production of IL-19 (Fig. 4B) in nasal epithelial cells.

We next explored whether r IL-19 was able to alter cell proliferation in nasal epithelial cells and whether cell proliferation was affected by herbimycin and by Na orthovanadate,

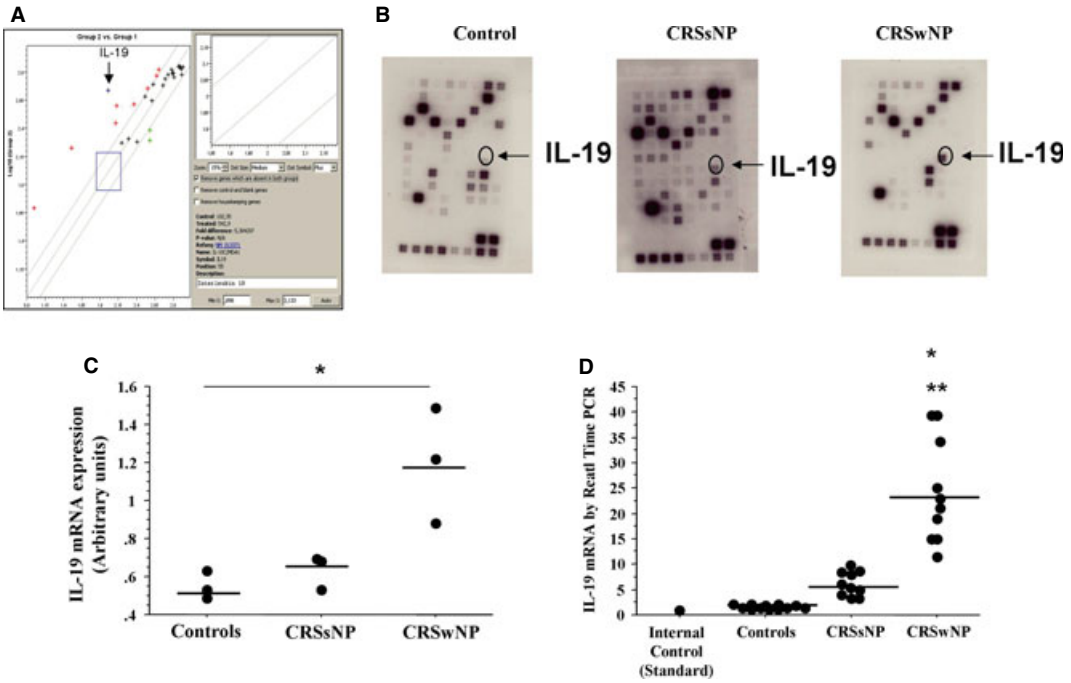


Figure 1 Increased expression of IL-19 mRNA in chronic rhinosinusitis (CRS) with nasal polyps by Human Asthma Gene Array. Nasal biopsies were collected from controls ($n = 3$), from subjects with chronic rhinosinusitis without nasal polyps (CRSsNP) ($n = 3$) and from chronic rhinosinusitis with nasal polyps (CRSwNP) ($n = 3$). Total RNA was extracted, and Human Asthma Gene Array was used to compare the gene expression profiles of the collected samples. (A) Representative analysis performed by the GEarray analyzer program showing the differences in gene expression of a control (group 1) and of a CRSwNP (group 2). (B) Representative membranes from control, CRS and CRSwNP patients. (C) Densitometric analyses of multiple GEarray experiments ($n = 3$). *$P < 0.05$. Individual values are shown. Horizontal bars represent median values. (D) IL-19 m-RNA expression was also assessed by real-time PCR in controls ($n = 12$), CRSsNP ($n = 10$) and CRSwNP ($n = 10$). GAPDH gene expression was used as endogenous control for normalization. Relative quantitation of mRNA was carried out with comparative CT method. Individual values are shown. Horizontal bars represent median values. * $P < 0.002$ *vs* controls; ** $P < 0.002$ *vs* CRS.

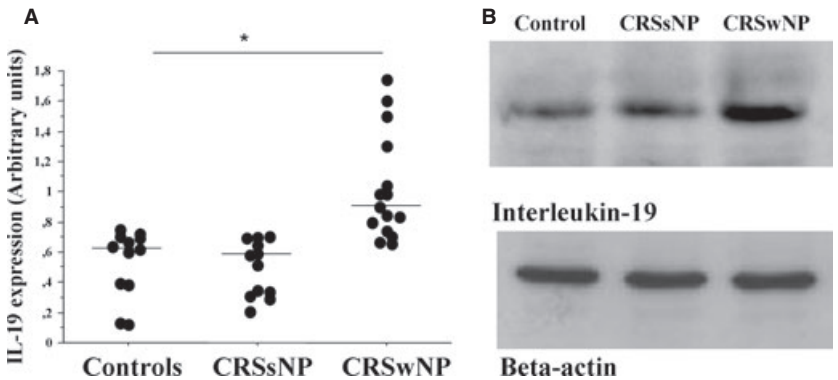
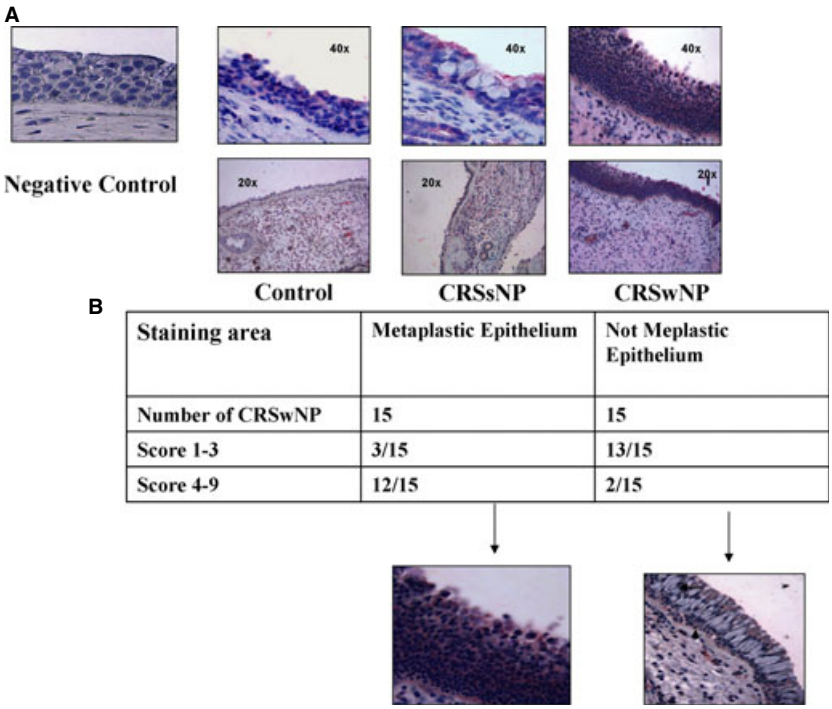


Figure 2 Increased expression of IL-19 protein in chronic rhinosinusitis (CRS) with nasal polyps by western blot analysis. Nasal biopsies were collected from controls ($n = 12$), from CRSsNP ($n = 12$) and from CRSwNP ($n = 15$). Total proteins were extracted and analysed for IL-19 expression by western blot analysis. Membranes were then stripped and incubated with goat polyclonal anti-ß-actin. (A) Densitometric analysis of IL-19 expression. Signals corresponding to IL-19 on the various western blots were semiquantified by densitometric scanning, normalized and expressed after correction with the density of the band obtained for β-actin. Individual values are shown. Horizontal bars represent median values. * $P < 0.05$. (B) Representative western blot analysis for IL-19 expression from a control, a CRS and a CRSwNP.



Staining area	Metaplastic Epithelium	Not Meplastic Epithelium
Number of CRSwNP	15	15
Score 1-3	3/15	13/15
Score 4-9	12/15	2/15

Figure 3 Higher expression of IL-19 protein in nasal epithelium of chronic rhinosinusitis (CRS) with nasal polyps. IL-19 expression was assessed by immunohistochemistry in controls ($n = 12$), CRSsNP ($n = 12$) and CRSwNP ($n = 15$). Negative controls were performed using rabbit immunoglobulins negative control. (A) Representative negative control and representative immunostaining (red stain) in a control, a CRSsNP and a CRSwNP at 200× and at 400× magnification. (B) The expression of IL-19 was assessed in metaplastic and in not metaplastic (normal and hyperplastic) epithelium of CRSwNP ($n = 15$). A composite score was used to evaluate IL-19 expression. At the bottom of the table, representative immunostainings are shown. Arrow points to mucous cell hyperplasia while arrowhead points to basal cell hyperplasia.

an inhibitor and an activator of tyrosine kinases, respectively. Recombinant IL-19 alone and with Na orthovanadate increased while herbimycin reduced the IL19-induced cell proliferation (Fig. 4C,D).

Squamous metaplastic nasal epithelium shows higher expression of Ki67 protein in CRS with nasal polyps

We finally investigated whether in the squamous metaplastic epithelium, a deregulated expression of Ki67, a marker well correlated with altered cell proliferation (20), occurred. The percentage of Ki67-positive cells was significantly higher in the metaplastic epithelium than in normal epithelium of nasal polyps from CRSwNP (Fig. 5A). Flow cytometry (Fig. 5B) in primary nasal epithelial cells from CRSwNP and immunofluorescence in CRSwNP samples (Fig. 5C–E) showed that Ki67 co-localized with IL-19 in nasal polyps.

Discussion

This study provides compelling evidence on the role of IL-19 in patients with CRSwNP. We have demonstrated that IL-19 induces nasal cell proliferation, is up-regulated at both mRNA and protein levels and is highly expressed by the squamous metaplastic epithelium of nasal polyps where an increased expression of Ki67 occurs.

CRS is an inflammatory disease with distinct cytokine and remodelling patterns. CRSwNP is characterized by a Th2-skewed eosinophilic inflammation, whereas CRSsNP represents a predominant Th1 milieu. CRS severely affects patients' quality of life and complicates inflammation in adjacent organs and worsens associated diseases including asthma (21). On the basis of the 'one-airway hypothesis', we initially approached this study using an Asthma Gene Array to explore the presence, in nasal compartment, of asthma like gene expression alterations. Asthma gene Array demonstrated that IL-19 is up-regulated, and real-time PCR analysis confirmed that IL-19 gene expression was increased in CRSwNP in comparison with normal subjects. Furthermore, IL-19 protein expression was increased in the epithelium of CRSwNP in comparison with normal subjects.

The genes encoding IL-19 and IL-20 cluster into the genomic IL-10 region on human chromosome 1 (22). Microarray technology and an immunohistochemical survey with an anti-IL-19 antibody to examine the expression of IL-19 in healthy and neoplastic tissues demonstrated that IL-19 protein was positively stained in healthy lung tissue by epithelial cells, endothelial cells and macrophages (23).

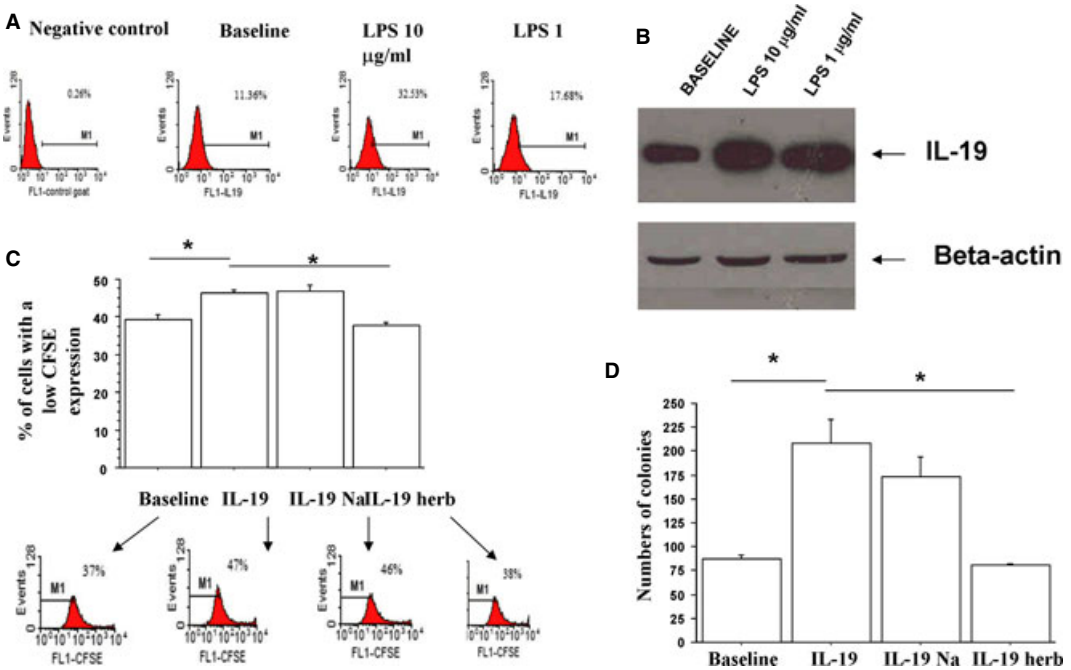


Figure 4 IL-19 expression and cell proliferation in nasal epithelial cells. Flow cytometric analysis (A) and western blot analysis (B) were used for assessing IL-19 expression in a nasal epithelial cell line (RPMI 2650) without or with LPS (1 and 10 µg/ml) stimulation. (A) Representative histograms of a flow cytometric analysis showing on the left the negative control using goat immunoglobulins negative control, and on the right, the expression of IL-19 in the absence and in the presence of stimuli. (B) Total proteins were extracted and analysed for IL-19 expression by western blot analysis. Membranes were then stripped and incubated with goat polyclonal anti-ß-actin. Representative western blot analysis is shown. Nasal epithelial cell line (RPMI 2650) was cultured with or without r IL-19 (200 ng/ml)and with IL-19 and herbimycin and Na orthovanadate. Cell proliferation was assessed using carboxyfluorescein succinimidyl ester (CFSE) by means of flow cytometry (C) and by clonogenic assay (D) (see Materials and Methods for details). (A) Means ± SD of $n = 3$ experiments. *$P < 0.05$ paired *t*-test. At the bottom of the panel A, one representative experiment is shown. (B) Means ± SD of $n = 3$ experiments. *$P < 0.05$ paired *t*-test.

There are limited comprehensive surveys of IL-19 expression in nasal human tissue or nasal cell types, and only few biological functions and clinical implications of IL-19 are known. It has been previously demonstrated that IL-19 down-regulates IL-4-induced eotaxin production in human nasal fibroblasts from patients with allergic rhinitis, (24) but no study specifically investigates the role of IL-19 in nasal epithelium and in CRSwNP.

The synthesis of IL-19 is a tightly regulated mechanism. In the present study, for the first time, a relevant increased expression of IL-19 in nasal epithelial cells was observed after LPS stimulation. In this regard, it has been demonstrated that TLR4 signalling pathway, after LPS stimulation, activates MyD88 and in turn induces Il-19 expression in monocyte cultures (25,26). Moreover, it has recently been demonstrated that nasal epithelial cells stimulated with enterotoxins B from *Staphylococcus aureus* release chemokines including IL-8 and IP-10 (27) (Huvenne Allergy 2010). Future studies are needed to clarify whether enterotoxins B from *S. aureus* or Th2 cytokines (IL-4, IL-13) are able to induce IL-19 expression/release. The increased levels of IL-19 in CRSwNP may actively contribute to amplify the inflammatory responses in these patients. IL-19, produced by synovial cells, promotes joint inflammation in rheumatoid arthritis by inducing IL-6 production and decreasing synovial cell apoptosis (28). IL-6 activates Th17 cells and regulates the response of B lymphocytes and of regulatory T cells. Consistently, in CRSwNP compared with controls, IL-6 and IL-6 receptor (29) are increased while Foxp3 mRNA, a transcription factor, typically associated with T regulatory activities was reduced (30,31). Furthermore, the increased release of IL-19 may alter the balance of Th1 and Th2 cells in favour of Th2 cells because this cytokine up-regulates IL-4 and down-regulates interferon-c (32,33). In bronchial asthma, a disease associated with increased Th2 responses, IL-19 levels are elevated three- to fourfold (33). High IL-19 levels correlate with high IL-4 and IL-13 levels, and epithelial cells in the airway of human asthma and patients with chronic obstructive pulmonary disease are rich in IL-19 (34). Accordingly, in CRSwNP, IL-5, IL-13 and GATA-3, typically associated with Th2 responses, are increased (35), and here, we demonstrated that in these patients also IL-19 expression is increased. A positive feedback between the Th2 and IL-19 in asthma has been demonstrated because bronchial epithelial cells stimulated by IL-17 and IL-13 express IL-19 at high levels (34). The

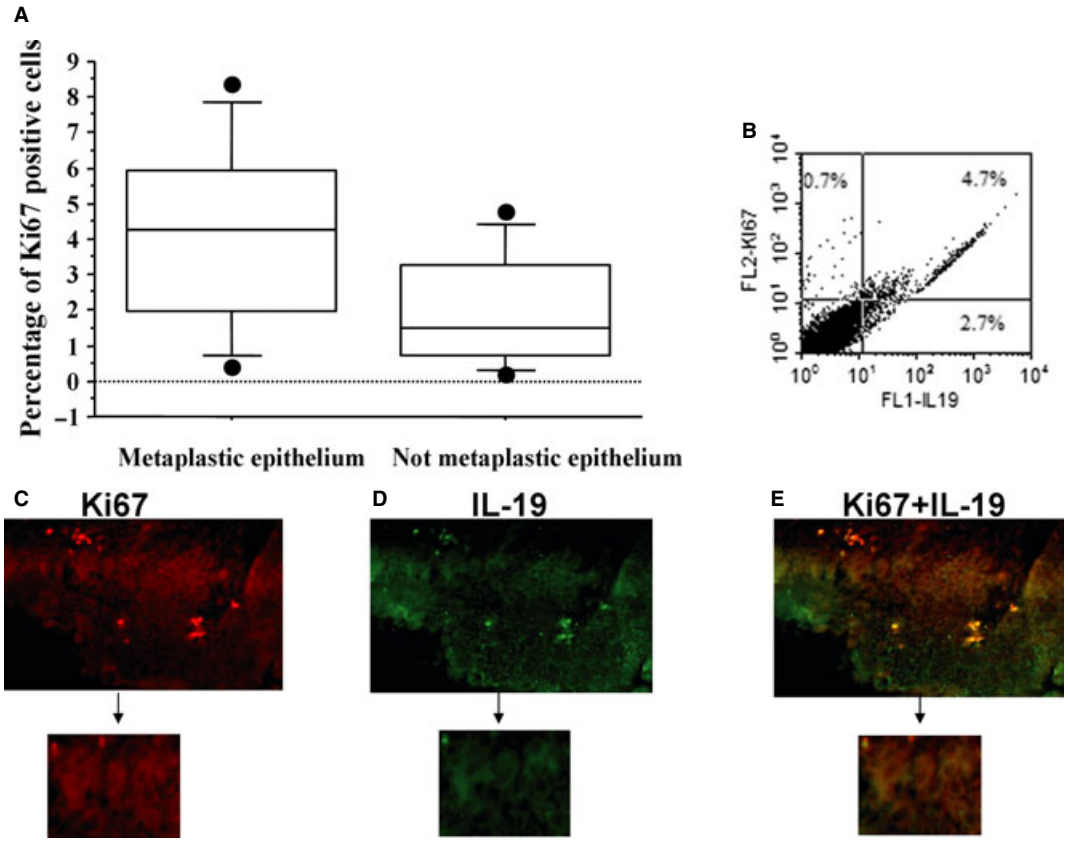


Figure 5 Squamous metaplastic nasal epithelium showed higher expression of Ki67 protein in chronic rhinosinusitis (CRS) with nasal polyps. (A) Immunohistochemistry was performed for assessing Ki67 expression in nasal biopsies collected from CRSwNP patients ($n = 15$). (B) Flow cytometry was performed for assessing the expression of Ki67 and IL-19 on nasal epithelial cells isolated from CRSwNP. A representative experiment is shown. (C–E) Immunofluorescence was performed for assessing Ki67 (red fluorescence) and IL-19 (green fluorescence) expression in nasal biopsies collected from patients with CRS and NP (CRSwNP) ($n = 3$). A representative experiment is shown. (C) Single immunofluorescence for Ki67. (D) Single immunofluorescence for IL-19. (E) Merged immunofluorescence for IL-19 and Ki67. For single ki67 and IL-19 and for double ki67/IL-19, a particular from a higher magnification is shown. See Materials and Methods for details.

presence of a similar mechanism also in patients with CRSwNP may be supposed.

In addition, the increased levels of IL-19 may alter the proliferation and the differentiation of the nasal epithelium in CRSwNP. In psoriatic skin, IL-19 induces a gene expression profile consistent with inflammatory responses, wound healing re-epithelialization and altered epithelial differentiation (36). Epithelial damage and aberrant tissue remodelling are common features of CRSwNP. Features of nasal remodelling may be considered: squamous metaplasia, basement membrane thickening, collagen deposition, hyperplasia of mucous glands and goblet cells (37). Here, squamous metaplastic nasal epithelium showed higher expression of IL-19 protein. Nasal epithelial cells from patients with nasal polyps demonstrated faster growth rates, and VEGF regulates nasal epithelial cell proliferation because exposure of cells to blocking antibodies against VEGF resulted in the inhibition of cell growth (38). IL-19 specifically activated an intracellular signal and induced proliferation in oral cancer cells (23). The binding of IL-19 to its receptor complex activates, as main pathways, the tyrosine phosphorylation and the signal transducers and activators of transcription (STAT) pathways, notably STAT1 and STAT3 (26,39,40). In the present study, IL-19 increased nasal epithelial cell proliferation, and herbimycin, a tyrosine kinase inhibitor, reduced IL-19-induced nasal epithelial cell proliferation, suggesting that the proliferative role of IL-19 in nasal epithelial cells is mediated by tyrosine phosphorylation events. Furthermore, IL-19 maximally activates tyrosine phosphorylation because the presence of Na orthovanadate, a phosphotyrosine phosphatases inhibitor, did not further increase cell proliferation. Finally, squamous metaplastic nasal epithelium and primary nasal epithelial cells from patients with CRSwNP highly co-expressed IL-19 and Ki67. Ki67 is vital for cell proliferation, and patients with CRSwNP and higher Ki67 planoepithelial metaplasia are the subgroup with eosinophilic infiltrations (41). Additional studies are needed to clarify the role of IL-19 in the Th2 bias and development of nasal polyps.

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Conflicts of interest

Jean Bousquet has received honoraria for scientific and advisory boards, lectures during meetings, press conferences from Stallergènes, Actelion, Almirall, AstraZeneca, Chiesi, GSK, Merck, MSD and Novartis and all other authors declares no competing interests.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Methods.

Table S1. List of 85 genes present in Asthma Gene Array.

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