

Polyp-promoting factors are expressed in polyp derived cell culture up on IL-4 and IL-13 treatment

Kathleen Susat

Klinikum Bielefeld-Mitte, Medical School OWL, Bielefeld University

Philipp Kühnel

Klinikum Bielefeld-Mitte, Medical School OWL, Bielefeld University

Claudia Scherl

Klinikum Bielefeld-Mitte, Medical School OWL, Bielefeld University

Matthias Schürmann

`matthias.schuermann@klinikumbielefeld.de`

Klinikum Bielefeld-Mitte, Medical School OWL, Bielefeld University

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Abstract

Background

Chronic rhinosinusitis with nasal polyps is driven by cytokines IL-4 and IL-13; their role in the establishment of polyp-promoting niche is not completely understood. This study aimed to identify key paracrine and autocrine factors induced by IL-4 and IL-13 in fibroblasts and epithelium.

Methods

A comprehensive literature search identified candidate factors implicated in CRSwNP pathogenesis, inducible by IL-4 and IL-13 (11 in fibroblasts / 14 in epithelium). Differentiated polyp epithelial cells (n = 6) and polyp fibroblasts (n = 4) were stimulated with IL-4 or IL-13; gene expression was quantified by qPCR. Epithelial barrier integrity was evaluated by transepithelial electrical resistance and qPCR in monocultures and co-cultures with patient-matched fibroblasts.

Results

In fibroblasts, IL-4 and IL-13 consistently upregulated POSTN, CCL11, CCL26, and TNC. In epithelial cultures, POSTN showed upregulation following IL-4 treatment, and CCL26 reacted to both cytokines. Other literature-derived factors exhibited inconsistent responses across biological replicates. Co-culturing ALI cultures with fibroblasts caused a decrease in epithelial integrity, particularly by IL-13. Downregulation of tight junction markers (ZO-1, Claudin-1, Claudin-3) and increased expression of fibroblast-derived FGF2.

Conclusion

It can be assumed that epithelial and fibroblastic cells exposed to IL-4 and IL-13 play a significant role in the increased eosinophilia (CCL11/CCL26) and tissue remodeling (TNC/POSTN) in polyp development. Keywords: primary cell culture model, CRSwNP, Th2-environment, epithelial-mesenchymal interaction, paracrine and autocrine factors

Introduction

CRS in general

Chronic Rhinosinusitis (CRS) affects 12% of the global population annually, making it one of the most common health issues worldwide (1–3). It involves inflammation of the sinuses and nasal mucosa, with symptoms such as facial pain, pressure, headaches, and cough, which all significantly impair the quality

of life (4–6). Treatment options currently include steroids, antibiotics, surgery, and most recently biologics (7–9).

CRS can be divided into two phenotypes: CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). CRSwNP tends to be more persistent and recurrent compared to CRSsNP (4, 10). This classification is essential for further precision in terms of diagnostic and the subsequent determination of optimal therapeutic interventions (11, 12). While CRSwNP is primarily characterized by Th2-mediated type 2 inflammation with elevated levels of IL-4, IL-5, and IL-13, as well as marked eosinophilic infiltration. CRSsNP tends to exhibit a Th1/Th17-associated response. It is characterized by neutrophils and pro-inflammatory cytokines such as TNF- α and IFN- γ (13, 14).

IL-4 and IL-13 signaling in the context of polyp growth

CRSwNP patients show a Th2 dominant immune response. Patients with high IL-4/IL-13 activity (type 2) typically show severe forms of progression and often with recurrence (15, 16). Clinical blockage of IL-4/IL-13 signaling results in a direct reduction in polyp growth compared to the control cohort (17). Thus it is widely accepted, that IL-4 and IL-13 are key drivers in the pathogenesis of the majority of CRSwNP cases (4, 18). On cellular level they promote the switch from B-cells to plasma cells and hence IgE synthesis, enhance eosinophil recruitment and mast cell degranulation (4, 19, 20). Additionally, they prolong survival of eosinophils in tissue and increases their inflammatory activity (18, 21).

The role of fibroblasts in polyp formation/Th2

In the context of polyp formation, fibroblasts are known to promote structural organization as well as inflammatory signaling (22, 23). Transcriptional analysis as well as proteome analysis showed that fibroblasts treated with type 2 cytokines IL-4 or IL-13 showed upregulation of genes associated with ECM remodeling (24, 25), as well as inflammatory signaling and fibroblast activation (26, 27). As sentinel cells, they play a key role in tissue remodeling through the excessive production of ECM and also enhance the Th2 inflammatory response by releasing chemoattractant molecules (24, 28). An increased expression of matrix metalloproteases (MMPs) was also shown, which play a role in matrix remodeling. This promotes fibrotic tissue structure, which is typically in polyps (29).

Epithelium in polyp formation/Th2

Epithelial cells vice versa contributing to polyp formation by their weakened barrier properties in polyps (19). This is caused by the reduced expression of tight junction proteins (30, 31), resulting in microbes or allergens easily passing through the barrier fueling inflammation (14).

Dysregulation of epithelial function by Th2 signaling is a key pathophysiological mechanisms of the inflammatory environment (32–35). Epithelial alarmins such as TSLP, IL-25, and IL-33 are known to be upregulated by pathogens/allergens particularly in Th2 environment. These cytokines promote type 2 immune response via activation of innate lymphoid type 2 cells and promotion of eosinophil inflammation. They play a key role in the maintenance of chronic inflammation in polyps (36–38).

Transcriptome and proteome analysis of epithelial cells treated with type 2 cytokines IL-4 or IL-13 showed also a significant change in expression of genes that are associated with remodeling (39), proliferation (40, 41), mucus production (42), and epithelial-to mesenchymal transition (EMT)(4, 43–45).

General interaction between fibroblasts and epithelium in TH2 associated diseases

But besides all this contribution to the pathogenesis of CRSwNP stromal and epidermal cells they play a major role as first line of defense in immune response, e. g. by communicating directly via paracrine signaling (11, 14, 37). For CRSwNP the epithelial mesenchymal interaction led to restricted differentiation of epithelial cell cultures compared to healthy control (46). More investigations were undertaken on other Th2 driven diseases. Fibroblasts derived from atopic dermatitis contribute particular to microenvironment of keratinocytes through paracrine mediators inducing hyperproliferation and reduced expression of differentiation markers (47). In the context of keloids, interaction between fibroblasts and keratinocytes resulted in strong fibroblasts proliferation and massive ECM production (48). Co-cultivation of epithelial cells and fibroblasts, isolated from asthmatic patients resulted in decreased proliferation of epithelial cells (49). It was demonstrated, that these fibroblasts achieved an increased expression of ECM associated genes (50). Also, co-cultivation with airway myofibroblasts and epithelial cell line revealed an increased proliferation of asthmatic myofibroblasts (51).

Aim

In recent years, the biologic Dupilumab, which inhibits IL-4 and IL-13 signaling, has been introduced into clinical practice for the treatment of recurrent CRSwNP. This study aims to identify fibroblastic and epithelial paracrine and autocrine factors (PAFs) that may be involved in CRSwNP pathogenesis, focusing on those previously linked to the disease and inducible by IL-4 or IL-13 in respiratory disease models. We will evaluate these candidate PAFs using a cell culture model derived from nasal polyp tissue and examine their functional effects in a co-culture system. The aim is to identify the PAFs, which are most robustly induced and hence considered a general driver of Th2 induced polyp growth.

Methods

Literature Review

A systematic comprehensive literature search was conducted to find PAFs related to polyp growth. The identification of potential PAF was based on the following 3 steps:

1. factor known to be involved in pathogenesis of CRSwNP (associated with clinical outcome)
2. factor needs to be involved in the PAF-System between fibroblasts and epithelial cell, resulting in a pathogenic outcome related to CRSwNP.
3. The potential PAF should be inducible via IL-4 and/or IL-13 in experimental respiratory tract models

The search was conducted using Google Scholar and PubMed. Studies were included if they met the following criteria: published in peer-reviewed journals, available in English, listed on PubMed.

Exclusion criteria: Studies were excluded if they focused on non-human subjects, lacked sufficient methodological rigor. The search comprised all studies executed since 1990 ensuring the inclusion of all relevant research. Disagreements in study inclusion were resolved through discussion between KS, PK and MS. Potential limitations of the review process include the exclusion of non-English publication, limited access to full texts, or non-identified methodological limitations of included studies. Another limitation is that the use of fully differentiated respiratory epithelium remains a relatively new and not yet widely established technique. To ensure broader coverage of the reviewed PAFs, we also included numerous studies using epithelial cultures under submerged conditions, which more closely resemble a population of epithelial stem cells rather than fully differentiated respiratory epithelium.

To identify potential factors involved in the pathogenesis of CRSwNP, keywords were chosen to identify any factor known to be involved in the pathogenesis of CRSwNP. For this search terms “CRSwNP” or “nasal polyps” were combined “cytokine” and “chemokine” to identify relevant studies and derive PAFs.

To identify the potential PAFs involved in fibroblasts epithelial related signaling, we conducted the following literature review. We combined the following terms “Name of PAF”, “interaction”, “epithelial cells”, “fibroblasts”.

Finally the PAFs were narrowed down to the PAFs inducible via IL-4 and or IL-13. For this the search term were “Name of PAF”, “IL-4” or “IL-13”, “stimulation”, “epithelial cells”, “fibroblasts”. Since the corresponding experimental evidence must be derived from respiratory tract models we also added the terms “nasal”, “lung”, “respiratory”

Cell culture

Tissue samples utilized in this study were nasal polyps from patients with CRSwNP, which were surgically obtained during pansinus surgery at the ENT of the University Hospital Bielefeld Mitte and stored in 1x Phosphate Buffered Saline (PBS) on ice. Samples were collected after written informed consent was obtained from all patients according to local and international guidelines (Ethics Committee of the university hospital Ruhr-Universität Bochum in Bad Oeynhausen, Germany; 20108-397_4). Human nasal polyp tissue was cut into small pieces in a 10 cm Petri dish (Starlab GmbH) under sterile conditions using disposable scalpel No. 20 (Feather). The minced polyp was transferred into 7 ml of collagenase and incubated for 30 min with horizontal agitation at 60 rpm at 37°C in the water bath. Afterward, the solution was centrifuged for 8 min at 1300 rpm. The supernatant was removed; the pellet was resuspended in 3 ml of medium and cultured in a T-25 flask (Starlab GmbH).

To expand primary cultures of epithelial cells (abbreviated with SP), PneumaCult-Ex Plus Medium (StemCell Technologies) was used. It was supplemented with 2% (v/v) PneumaCult-Ex Plus 50X Supplement (StemCell Technologies). To expand primary culture of fibroblasts (shortened to SPDF), Dulbecco's Modified Eagle Medium (DMEM; Capricorn Scientific GmbH) was used. It was supplemented with 10% (v/v) fetal bovine serum and 2 mM L-glutamine (both Capricorn Scientific GmbH). When confluence of 85–100% was reached, adherent cells were detached with 3 ml Accutase 37°C and

pelleted at 300 rcf for 8 min. The supernatant was discarded, and the pellet was resuspended in medium. Afterward, cells were expanded into T-25 flasks or seeded in wells for further experiments. All cultures were kept under standard conditions (37°C with 5% CO₂) and all media were supplemented with 2.5 µg/ml amphotericin B, 1% (v/v) 100x penicillin-streptomycin (P/S) (both Capricorn Scientific GmbH).

For differentiation of the epithelial cells into mature epithelium at the air liquid interface (ALI), PneumaCult-ALI medium (StemCell Technologies) was applied. It was complemented with 10% (v/v) PneumaCult ALI-10X supplement and was stored as 33 ml aliquots at -20°C and thawed when needed. When ALI-Medium was thawed 4 µg/ml heparin, 1% (v/v) PneumaCult-ALI Maintenance 100x Supplement, and, 1 µM hydrocortisone (HC) were added. Medium was stored at 4°C for a maximum of 2 weeks. To establish an ALI culture, about 30,000 cells/well of passage 1 were seeded on 0.4 µm pore transwell inserts (SABEU GmbH & Co. KG, Germany) in a 12-well plate (Starlab, Germany). The apical side was filled with 500 µl, and the basolateral side was filled with 1 ml PneumaCult-Ex Plus Medium. The medium was changed every other day. The cells were incubated until 100% confluence. Subsequently, differentiation was induced by the exposure of the apical side of the culture to air and by changing the basolateral medium to 1 ml of ALI-Medium. The medium was changed every other day, and differentiation took 21 days. When mucus was formed by goblet cells, the cells had to be washed with 1x PBS once a week to remove mucus starting mostly after 14 days.

Culture Treatments

About 50,000 cells/well of SPDF cells of passage 1 to 4 were seeded on 6-well plate with 1 ml DMEM medium and the medium was changed every other day. When cells reached 100% confluence, they were starved with DMEM medium w/o FBS for three days before treatments were started. Before treatment, the terminal differentiated SP ALI cultures were incubated for three days with ALI Medium w/o HC.

Before treatment cells were washed with 1x PBS. The treatments were performed in medium w/o HC or serum respectively. Usually treatment with high dose of 100 ng/ml for SP ALI or 50 ng/ml for SPDF of IL-4 and IL-13 was performed for 24 h (unless otherwise stated). Cells not exposed to these cytokines served as medium control (MC). In some cases, the cells were also treated with these Th2 cytokines in combination with dupilumab. Two different experimental approaches were used. In one approach, the cells were first pretreated with 10 µg/ml dupilumab for one hour before 100 ng/ml IL-4 or IL-13 was added for a period of 24 h. In the alternative experimental setup, the cells were first stimulated with 100 ng/ml IL-4 or IL-13 for 18 h, followed by a further incubation with 10 µg/ml dupilumab for 6 h. The cells were incubated under standard conditions. During the treatment the cells might underwent non disturbing measurements (e. g. TEER measurements). To terminate the experiment, cells were lysed with lysis buffer for qPCR analysis.

For co-cultivation experiments, fibroblasts between passages 1 and 4 were seeded in 1 ml of DMEM supplemented with FBS into a 12 Well with 50.000 cells/well. Once the fibroblasts reached full confluence, both the fibroblasts and the corresponding patient-derived matured ALI cultures were

maintained for three days in ALI medium without hydrocortisone (w/o HC) prior to the experiments. At the start of the stimulation experiments, transwell inserts containing the matured ALI cultures were placed above the fibroblasts, and treatments were initiated immediately. Throughout the experiments, the cells were kept in ALI medium without HC under standard culture conditions.

Transepithelial Electrical Resistance (TEER) Measurement

For TEER measurements, the EVOM2TM with a STX4 EVOMTM electrode (World Precision Instruments) was used. Before measurement, the electrode was cleaned and sterilized with 70% isopropanol for 15 min. The medium was removed from the cells and 2000 µl TEER buffer consisting out of HEPES 20 mM (Capricorn Scientific GmbH), 0.9% NaCl, and 3 mM CaCl₂ was added to the lower chamber and 700 µl to the upper chamber. An insert w/o cells was used as a blank. The electrode was equilibrated in TEER buffer for 3 min. Afterward, the electrode was connected to EVOM2TM, and the instrument was set to OHMS function (~ 30 Ω). For measurement, the longer electrode was placed in the lower chamber and the shorter in the upper chamber. TEER values were measured 3 times for each sample. After measurement, the electrode was cleaned in 70% isopropanol for 5 min and afterward air-dried. The TEER value was calculated as follows:

$$TEER\ value\ (\Omega * cm^2) = (measured\ TEER\ value\ (\Omega) - blank\ value\ (\Omega)) * membrane\ area\ (cm^2)$$

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted with the innuPREP RNA mini kit (Analytic Jena GmbH + Co. KG), according to the manufacturer's guidelines and eluted with 40 µl H₂O. For determination of RNA quality and quantity, a nano photometer (Implen GmbH) was used. The Biozym cDNA kit (Biozym Scientific GmbH) was used for cDNA synthesis according to manufacturer's guidelines. Random hexamer primers and 1000 ng of RNA template were used. If RNA concentration was 90 ng/µl or less, 11.5 µl of RNA solution was used, which was the maximum applicable volume of RNA solution of the kit. The cDNA synthesis was performed by the Thermocycler Tgradient Thermoblock (Biometra GmbH) with the following steps: 10 min incubation at 30°C, 60 min incubation at 60°C, 5 min incubation at 95°C, cooling down to 4°C.

The real-time PCR was conducted in accordance with the manufacturer's protocol provided by the Luna® Universal qPCR Master Mix (New England Biolabs Inc). The cDNA was diluted at 1:10 and the primer concentration was set at 0.25 µmol. All Primers for targets used in this study are listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control to each sample. All reactions were performed in triplicates for determination of statistical significance. A MIC qPCR cycler (BioMolecular Systems) was used for amplification with 40 cycles, 95°C for 15 sec and 60°C for 30 sec. Data quality was determined using melting curve and clustering of triplicate. Differences in expression levels were identified using the $2^{-\Delta\Delta CT}$ method. Graphics were designed using GraphPad Prism (GraphPad Software).

Table 1

Overview of all primers, their targets, and nucleotide-sequence used for qRT-PCR. The primers were designed to have an amplicon size between 70 and 200 bp and an annealing temperature close to 60°C

ACTA2	CCCAGCCAAGCACTGTCA	IL-25	CTGGAGGCTGGTCCCTTTTT
	GCCATTGTCACACACCAAGG		CTGCTCCAGACAGCACTTCA
Claudin-1	GCTTCTCTCTGCCTTCTGGG	IL-33	CATGCCAACAACAAGGAACA
	TCACACGTAGTCTTTCCCGC		AGGACAAAGAAGGCCTGGTC
Claudin-3	AAGATCACCATCGTGGCAGG	MMP2	TGCATCCAGACTTCCTCAGGC
	TCCCGGATAATGGTGTGTTGGC		CCTGGCAATCCCTTTGTATGTTT
CCL2	AATCAATGCCCCAGTCACCT	MMP7	CATGATTGGCTTTGCGCGAG
	CTTCTTTGGGACACTTGCTGC		AGACTGCTACCATCCGTCCA
CCL5	CTGCTTTGCCTACATTGCCC	OPN	GCCGAGGTGATAGTGTGGTT
	TCGGGTGACAAAGACGACTG		AACGGGGATGGCCTTGTATG
CCL11	AACCAGAGCCTGAGTGTTGC	PDGF-AA	GTCCGCCAACTTCCTGATCT
	AAACCCATGCCCTTTGGACT		CTTGACACTGCTCGTGTTGC
CCL13	GGTTGCTGTCTCTAAGCCCC	POSTN	AAGAAGACACACCCGTGAGG
	CAACCCCTGGGAACCGAATA		ACGACCTTCCCTTAATCGTCT
CCL26	CCTGGGTGCGAAGCTATGAA	TGFβ1	CCGGTAGTGAACCCGTTGATGT
	CCTCTTTTGGTAGTGAATATCACAG		TCCTGGCGATACCTCAGCAA
FGF2	CTGGCTATGAAGGAAGATGGA	TGFβ2	CTCCGAAAATGCCATCCCGC
	TGCCCAGTTCGTTTCAGTG		GCTCAATCCGTTGTTTCAGC
GAPDH	CTGCACCACCAACTGCTTAG	TNC	TCTCGCCCATCGGAAAGAAA
	GTCTTCTGGGTGGCAGTGAT		CCATGGTGGAGGTGGGTTTG
IL6	GCAAAGAGGCACTGGCAGAAAACA	TSLP	TACTCTGTTGGTGGGAAGAG
	TTCTGCAGGAACTGGATCAGGACT		GCAAAAGGGAACATACGTGGA
IL-8	TCTCTTGGCAGCCTTCCTGATTTC	ZO-1	CCCGAGGAAATGATGAGGCA
	AGTTTTCTTGGGGTCCAGACAGA		GAGGTCTCTGCTGGCTTGTT

Results

Literature review PAF Fibroblasts

The literature review identified 13 distinct molecular PAFs that are produced by fibroblasts in response to IL-4 or IL-13 stimulation and have demonstrated effects on either fibroblasts or epithelial cells (Table 2). The accompanying table consolidates relevant studies for each PAF, providing details on cell type, treatment conditions, treatment duration, analytical methods, principal findings, and the corresponding references.

Of 27 publications, 15 used primary fibroblasts from nasal or lung tissue (10 nasal polyps, 5 lung). Most treatments lasted 24 h, though 8 experiments used different durations. IL-4 concentrations ranged from 2–100 ng/ml and IL-13 from 10–100 ng/ml, with 10 ng/ml most common (12 experiments). qPCR was the predominant analytical method, supplemented by RT-PCR, ELISA, western blot, or northern blot. All PAFs were produced by fibroblasts and acted on fibroblasts, while only CCL13, IL-6, POSTN, and TNC affected epithelial cells.

Since several of these PAFs have not previously been studied in primary nasal polyp–derived fibroblasts, we performed corresponding experiments. A relatively high cytokine concentration was used, as it yielded the most consistent responses. POSTN, CCL26, CCL11, and TNC were the most robustly upregulated by both cytokines.

POSTN expression was upregulated 5.5- to 26-fold following treatment with IL-4 or IL-13, with significant increases observed in all samples after at least one of the cytokine treatments. CCL26 expression was strongly induced, ranging from 99- to 3,371-fold, and showed significant upregulation in all samples, particularly after IL-13 treatment. CCL11 expression increased 2- to 42-fold and was significantly upregulated by both IL-4 and IL-13 in all samples. TNC expression was also elevated, with a fold change of 2.2 to 48; most samples showed significant upregulation after both cytokines, while some responded preferentially to IL-13.

In contrast, CCL2 and IL-6 showed partial regulation, with IL-6 responding more consistently to IL-13 and CCL2 more to IL-4 (Fig. 2). The remaining candidate PAFs did not exhibit reproducible responses to the treatments (Sub. Figure 1).

Literature review PAF Epithelia

The literature review identified 14 molecular PAFs expressed by epithelial cells in response to IL-4 or IL-13, which modulate fibroblasts or epithelial cells in the context of nasal polyp growth. Table 3 summarizes each PAF, detailing cell type, treatment conditions, duration, analytical methods, key findings, and references.

Of 23 studies, 16 used primary epithelial cells from nasal or bronchial tissue, including human bronchial epithelial cells (10 studies), tracheobronchial cells (3 studies), and alveolar type II cells, with only three derived from nasal tissue (1 nasal polyp, 2 HNECs). Six studies used cell lines, mainly A549 alveolar type II–like cells. Cells were cultured as submerged (11 studies), air–liquid interface (ALI; 5 studies), or both (6 studies). Treatment durations were mostly 24–48 h, though some were shorter, longer, or spanned

multiple days. IL-4 and IL-13 concentrations ranged from 0.1 to 150 ng/ml, with 10 ng/ml most common. Analytical methods primarily included qPCR, RT-PCR, and ELISA, with occasional western blot analysis.

Establishing pure epithelial cell cultures and ALI cultures was challenging and remains uncommon, and only one study has investigated the effects of IL-13 in CRSwNP ALI cultures.

All genes were screened in preliminary qPCR assays to assess reproducibility. Only POSTN, CCL26, CCL11, and CCL24 were successfully induced by 100 ng/ml IL-4 or IL-13 in six patients (Fig. 3). ALI cultures treated with IL-4 or IL-13 generally showed upregulation of POSTN, with fold changes ranging from 3 to 6,650, except for one IL-13-treated culture (0.6-fold). CCL26 upregulation was highly variable (1.2-1,764-fold), with significant increases after IL-4 in two patients. CCL11 expression ranged from 1.7-285-fold, though two cultures showed reduced expression after IL-13 (0.2-0.9-fold); IL-4 significantly increased CCL11 in two cultures and CCL24 showed only a slight and weakly consistent upregulation. Other PAFs (TGF β 2, IL-8, IL-33, HB-EGF, CCL5, and TSLP) showed no consistent induction. (Sub. Figure 2).

Co culture

TEER measurements were performed in ALI cultures, both as monocultures and co-cultured with fibroblasts from the respective donor. Treatments with IL-4 and IL-13 were applied for 48 hours, and TEER values were recorded before and immediately after treatment. Unlike the monocultures, all co-cultures showed a decrease in TEER after 48 hours compared to the medium control. Notably, IL-13-treated co-cultures exhibited a pronounced and statistically significant reduction. Additionally, TEER values were lower in both untreated co-cultures and those treated with IL-4 compared to the monoculture control. A downregulation of the tight junction protein ZO-1 in all co cultures to approximately 60–70% normalized to the respective monoculture was observed. SP ALI co cultures exhibited also reduced reduced expression of tight junction transcripts Claudin-3 (monoculture and IL-13) as well as Claudin-1 (IL-4 and IL-13) to around (30–40%). In fibroblasts of co cultures FGF2 was induced by treatment with IL-4 and IL-13 by about 3 fold.

Discussion

Determine Stimulation of PAFs Secreted by SPDFs

Among the most robustly induced genes were POSTN, CCL26, and CCL11, all of which showed significantly increased expression following 24 hours of IL-4 or IL-13 treatment compared to controls. These findings are consistent with previous studies using comparable or lower cytokine concentrations executed with SPDFs for CCL11 and CCL26 but the first time this only observed in this working group for POSTN in SPDFs. POSTN, a matricellular protein involved in extracellular matrix organization and epithelial cell proliferation, is highly responsive to IL-4 and IL-13, typically reaching maximal expression after 24 hours of stimulation(52). CCL26, a potent eosinophil chemoattractant characteristic of Th2-driven polyp growth, was similarly induced, aligning with prior observations in SPDFs and HLFs, where 10

to 80-fold increases were reported depending on cytokine concentration and exposure time (53, 54). CCL11, another eosinophil chemoattractant, showed consistent upregulation compared to earlier studies in which 10 ng/mL IL-4 enhanced expression (55, 56). Notably, prior studies did not observe IL-13-induced CCL11 upregulation; however, the lower IL-13 concentrations used (one tenth of this study's dose) likely account for this discrepancy. This concentration-dependent regulation suggests that CCL11, like POSTN and CCL26, reaches maximal expression at higher IL-4 and IL-13 doses, highlighting its key role in type 2 cytokine-driven inflammation.

Compared to these chemokines, CCL2 exhibited a smaller increase after IL-4 or IL-13 treatment, this is in accordance with previous studies reporting only 2 to 4-fold induction in fibroblast cell lines and primary lung fibroblasts (53, 57). In contrast, fetal lung fibroblast lines such as GM06114 showed no induction, highlighting the influence of cellular origin and differentiation state on cytokine responsiveness (58).

The extracellular matrix-related gene TNC was significantly upregulated 2 to 10-fold after IL-4 or IL-13 treatment. The expression of TNC in SPDFs was never investigated so far. While fetal lung fibroblast lines (MRC5) did not show TNC induction after 10 ng/mL IL-13 treatment (52), primary dermal fibroblasts demonstrated similar IL-4 or IL-13 induced upregulation as observed here (59, 60). These differences likely reflect limitations of utilizing cell lines as model for the in vivo situation that could be attributed to variation in cytokine receptor expression.

TNC is implicated in tissue remodeling and epithelial-to-mesenchymal transition in multiple diseases, like atopic dermatitis (61) and most importantly CRSwNP, suggesting that its regulation in SPDFs mirrors disease-related fibroblast activation (62, 63).

IL-6 upregulation displayed donor-dependent variability: One responded only to IL-13 in a consistent manner. This partially contradicts previous reports, where IL-4 was a more steady inducer at both mRNA and protein levels (57, 64). However, prior studies either used cell lines or lower IL-13 concentrations (10 ng/mL), which may have been insufficient to trigger IL-6 induction in those contexts.

Some candidate mediators, including CCL5, CCL13, TGF β 1, and TGF β 2, showed inconsistent regulation or lacked clear expression trends (Sup. Figure 2). The variable response of CCL5 contrasts with prior findings in HLFs, possibly due to longer stimulation times (48 hours) or the disease-specific phenotype of SPDFs derived from CRSwNP tissue. CCL13 was generally expressed on a low level and hardly inducible by IL-13 treatment. Previous studies reported IL-4-induced upregulation in SPDFs, which we could verify (55, 65), highlighting cytokine-specific effects and importance of disease specific primary cell cultures. No significant changes were observed for the TGF β isoforms, although earlier reports described IL-4 responsiveness in SPDFs treated with 10 ng/mL for 24 hours in a not quantitative manner via RT PCR (55). SPDF cultures revealed no significant regulation of PDGF-AA through IL-4 or IL-13. However Ingram et al., showed, that IL-13 was able to induce PDGF-AA expression in a human fibroblastic lung cell line promoting their proliferation (66). This divergence in results might be attributed to either species-specific regulatory mechanisms or inherent differences in the phenotypic responses of fibroblasts derived from different anatomical sites.

Overall, not all literature-reported effects could be reproduced, but this could be explained by differences in detection methods (qPCR versus RT-PCR, WB or ELISA), fibroblast type (primary versus immortalized, diseased versus healthy, adult versus fetal), and cytokine responsiveness. But despite this unsuccessful translation of these PAFs into the CRSwNP context, this study showed for the first time the induction of POSTN and TNC in SPDFs. In general, the results demonstrate that SPDFs are responsive to IL-4 and IL-13, expressing mediators relevant for epithelial cell response in CRSwNP. The consistent upregulation of POSTN, CCL26, CCL11, and TNC underscores their role as central paracrine signals in type 2 inflammation, while the variability of other genes highlights donor-specific and context-dependent fibroblast behavior. These findings emphasize the importance of using disease-relevant primary cells and carefully defined stimulation protocols when investigating fibroblast-epithelial interactions.

Determine Stimulation of PAFs Secreted by SP ALIs

The aim of this study was to identify potential epithelial-derived mediators that are induced by IL-4 or IL-13 and may subsequently affect fibroblasts. Based on a comprehensive literature review, 15 candidates were selected that are known to be regulated by IL-4 or IL-13 in epithelial cells and play a role in CRSwNP pathophysiology (Table 2). To validate their relevance in the model system used in this study, expression analyses were performed via qPCR (Fig. 3).

SP ALI cultures showed increased expression of POSTN after IL-4 treatment, a target not investigated in SP ALI cultures so far, while IL-13 treatment did not produce a reproducible effect across biological replicates. These findings are only partly consistent with previous studies in HBECs, HNECs, TBE, and ATII cells, which reported similar upregulation of POSTN after IL-4 but also IL-13 stimulation regardless of the culture model (33, 33). Particularly a study on HNECs cultured as ALIs, showed a 10 to 15-fold increase in POSTN expression after the same treatment we applied. It is important to note that these studies used cells derived from healthy nasal tissue rather than polyps, which may inherently affect gene expression. Hence we suggest, that POSTN is a robust type 2-responsive mediator in differentiated cells and particularly susceptible to IL-4 in cell derived from CRSwNP.

CCL26 expression was significantly increased after IL-4 treatment in SP ALI cultures. After IL-13 treatment, only a trend toward increased expression was observed in SP ALI, but no statistical significance was reached in all samples. These results align with previous studies in primary respiratory epithelial cells such as HBECs and ATII cells, where IL-4 or IL-13 stimulation increased CCL26 at both mRNA and protein levels under ALI and submerged conditions (33, 67, 68). Comparable upregulation was also observed in the A549 respiratory epithelium-like cell line after IL-4 treatment (69). These findings indicate that CCL26 is a consistently regulated chemokine with potential roles in eosinophil recruitment and fibroblast activation (70), also in a CRSwNP derived model.

CCL11 expression in SP ALI cultures showed only a weak and not always significant increase after IL-4 treatment, with no consistent trend observed after IL-13 treatment. These results are in line with the study comparing HNECs, HBECs and TBE, which could only detect an upregulation in the lower respiratory tract (71).

CCL24 showed increase in expression after IL-4 or IL-13 treatment of SP ALI cultures but in a very inconsistent manner. Although contradictory data from the lower respiratory tract does exist (67), we suggests that CCL24 may serve as a minor but consistent type 2-responsive mediator in the nasal region.

TGF β 2 expression was unchanged in SP ALI cultures after IL-4 or IL-13 treatment. This pattern mirrors previous findings in HBECs, where upregulation was detected only in submerged cultures, not in ALI cultures (44). TGF β 2 expression has also been shown to increase at low cytokine concentrations (0.1 to 20 ng/mL) in ELISA studies(72, 73), indicating that epithelial cell differentiation state critically affects TGF β 2 expression.

No significant transcriptional changes were observed for IL-8 after IL-4 or IL-13 treatment, although other studies reported protein-level upregulation in the lower respiratory tract (35, 74). IL-33 expression decreased slightly after IL-4 treatment and remained largely unchanged after IL-13 treatment, differing from studies in A549 cells that showed IL-13-induced upregulation after 72 hours (36). Differences in treatment duration and cell type likely explain this discrepancy. HB-EGF expression remained unchanged after IL-4 or IL-13 treatment. Previous studies in 1HAEo- cells reported a three-fold increase in HB-EGF protein levels after IL-13 treatment (75). This discrepancy may be due to post-transcriptional regulation, differences between mRNA and protein detection, or the use of cell lines rather than primary cells. CCL5 expression showed no trend after IL-4 or IL-13 treatment, even though previous studies observed increase in protein levels but only in A549 cells (76).

Treatment of SP-ALI cultures with IL-4 and IL-13 resulted in inconsistent regulation of IL-25 expression. While studies on human lung tissue cultures describe a clear upregulation of IL-25 by IL-4 (77), this effect could not be reproduced in the present data due to the high variability between experimental series. Since there is also no data in the literature regarding IL-13-induced IL-25, the fluctuating results observed here underscore the difficulty of establishing a robust regulatory role of IL-25 in our CRSwNP model. The expression of IL-33 decreased unexpectedly following treatment with IL-4 and remained largely unchanged after treatment with IL-13. In contrast, previous studies reported an upregulation of IL-33 after 72 hours of IL-13 treatment in the A549 cell line (36). The discrepancy between these findings and the results of this study may be explained by differences in treatment duration and the use of a cell line versus primary tissue.

Overall, SP cells from CRSwNP patients exhibited a selective transcriptional response to IL-4 and IL-13. POSTN and CCL26 showed robust and consistent upregulation, particularly for stimulation with IL-4, highlighting their key role in epithelial-mesenchymal signaling under type 2 inflammatory conditions. Other mediators, such as CCL11 and CCL24, displayed partial or condition-dependent responsiveness, while TGF- β 1, IL-25, IL-33, IL-8, and CCL5 showed no significant transcriptional changes. These results underscore the complexity of cytokine responses in SP compared to other cell lines from literature. Limitations of this study were the rather short incubation time of 24 h. Even though this was the most commonly used time it prevent the insight into long term effects present on chronic inflammations. We

were also not able to detect the expression of GM-CSF and IGF-1 in epithelial cells with sufficient reliability to test its regulation. Another limitation was the focus on the RNA level. A Protein-level analysis, such as ELISA, may be necessary to fully understand the biological relevance of these mediators.

TEER Measurement

TEER measurements of ALI cultures were used as a functional assay to assess epithelial barrier integrity. Two patients were analyzed as both monocultures and co-cultures, with treatments of IL-4 or IL-13. In both patients, TEER values decreased in co-cultures under all conditions, with further reductions observed following IL-4 or IL-13 treatment in most cases, occasionally reaching statistical significance.

Fieux et al. reported a significant decrease in TEER in HNEC after 48 h treatment with IL-4 at 1, 10, or 100 ng/ml, consistent with the observations in this study (78). In contrast, they observed an increase in TEER following IL-13 treatment at the same concentrations. In our SP derived ALIs, IL-13 did not stabilize barrier function and instead caused the greatest impairment in co-cultures.

Other studies also report TEER reductions following IL-13 treatment, demonstrating barrier dysfunction not only through TEER measurements but also via decreased expression of tight junction proteins such as occludin, ZO-1, and CLDN3. We observed a similar effect on the expression of ZO-1, CLDN1 and CLDN3. Notably, these studies generally applied IL-13 for longer periods than the 48h applied here (79, 80). Importantly, no prior data on TEER measurements in CRSwNP derived co-cultures are available. The more pronounced barrier impairment observed in co-cultures is likely attributable to crosstalk with fibroblasts.

In summary, the in vitro findings obtained from primary cell cultures demonstrate that IL-4- and IL-13-mediated signaling contributes to the pathogenesis of CRSwNP by inducing pathogenic extracellular signaling in both epithelial and fibroblastic cells. This is characterized by the upregulation of cytokines that are well established as dysregulated in the immune response of patients with CRSwNP, including the eotaxins CCL11 and CCL26, as well as proteins implicated in the tissue remodeling associated with the disease, such as periostin and tenascin C. These findings further support their potential utility as biomarkers of IL-4/IL-13-driven CRSwNP. Furthermore, our results indicate that crosstalk between fibroblastic cells and the epithelium markedly compromises epithelial barrier integrity, an effect that is further exacerbated in the presence of IL-4 and IL-13 signaling. Possible this is due to increase of FGF2 expression in co cultivated fibroblasts, which is known to downregulate the expression of Cadherin-2 in epithelial cells (81) and Cadherin-1 (82) in epithelium of the lower respiratory tract.

Conclusion

In conclusion, this study demonstrates that primary nasal polyp-derived epithelial cells and fibroblasts exhibit transcriptional responses to the key type 2 cytokines IL-4 and IL-13.

Not all potential candidate factors showed the same response in polyp-derived cells. But POSTN, TNC, CCL11, and CCL26 emerged as central, regulated transcripts. These factors are known to contribute to hallmarks of polyp development like tissue remodeling, extracellular matrix degradation, and eosinophil recruitment. Additionally, our co-culture model reveals that epithelial-mesenchymal crosstalk alone plays a critical role in destabilizing mucosal integrity, an effect markedly exacerbated under IL-13 stimulation, presumably by fibroblast-derived FGF2.

Together, these findings underscore that CRSwNP pathogenesis relies not only on individual cellular responses to Th2 cytokines but also significantly on crosstalk between epithelium/fibroblasts and other cells.

Targeting these specific paracrine signaling pathways, particularly periostin, tenascin C, and eotaxins, presents a promising biological strategy to prevent polyp formation, e.g., through topical treatments of the mucosa.

Declarations

Acknowledgements and conflict of interest

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Data availability

All data are provided in this study, and raw data can be requested to the corresponding author.

The authors declare no competing interests.

Author Contribution

The authors contributed to the project in the following roles: K.S. was responsible for Methodology, Formal analysis, Investigation, Visualization, Data curation, Writing – original draft, Writing – review & editing, and Visualization, P.K. contributed through Methodology, Formal analysis, Investigation, Data curation, Supervision, Visualization, Conceptualization, Validation and Writing – original draft, Writing – review & editing. C.S. was involved in Resources, and Writing – review & editing, M.S. was involved in

Conceptualization, Methodology, Validation, Formal analysis, Resources, Writing – original draft, Writing - review & editing, Supervision, Project administration, and Funding acquisition.

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Tables

Table 2 and 3 available in the Supplementary Files section.

Figures

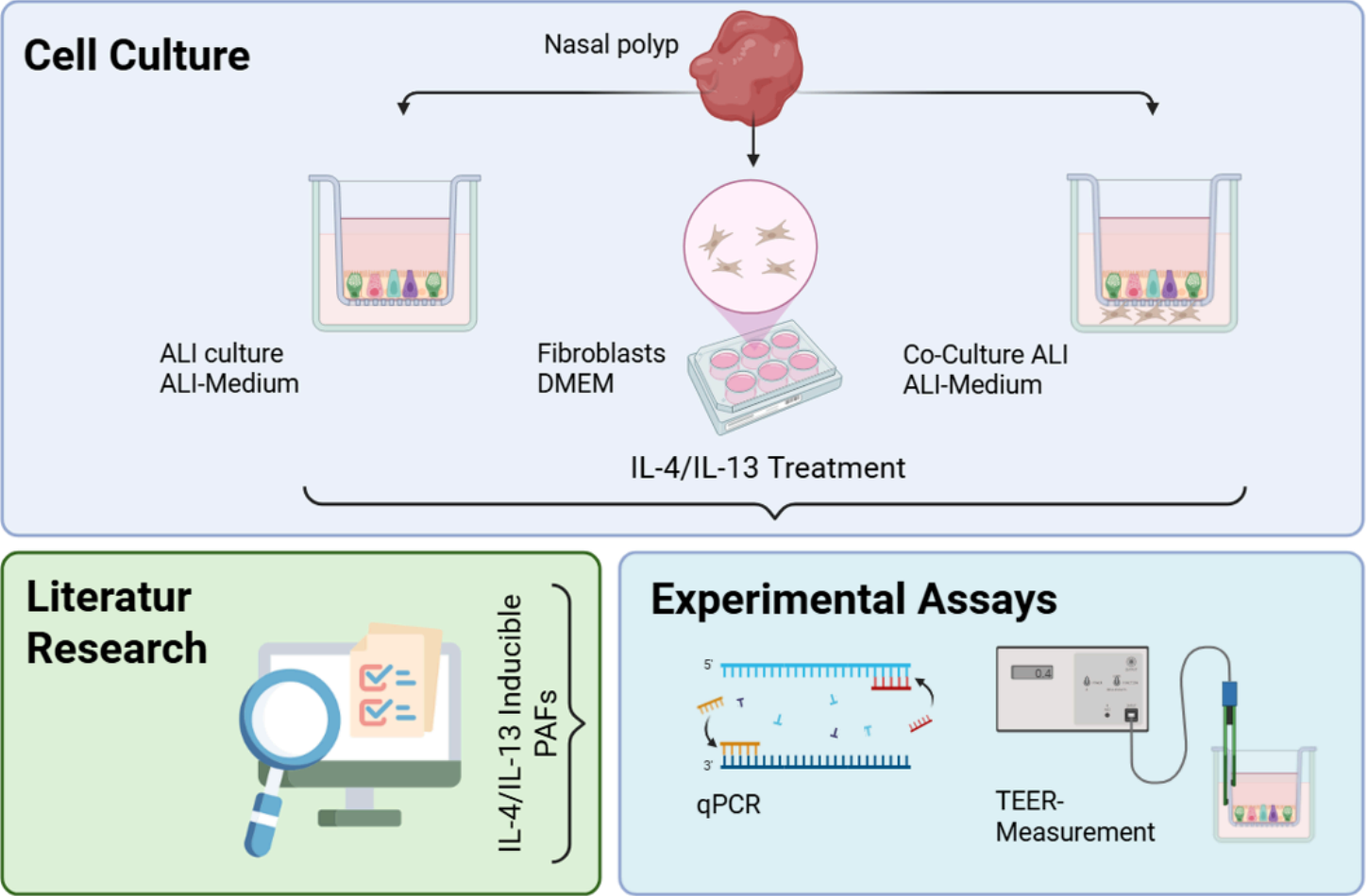


Figure 1

Overview of the general workflow of this study. The literature review was validated using primary cell cultures derived from nasal polyps

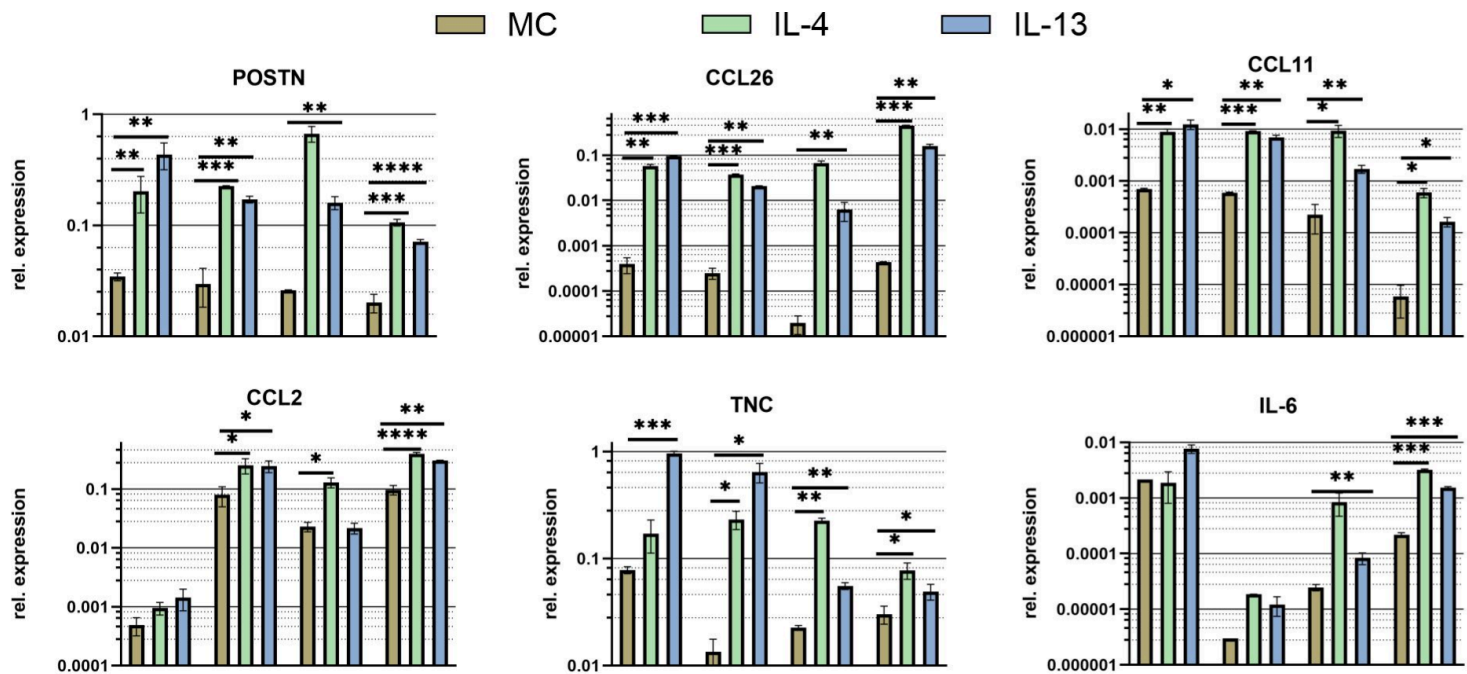


Figure 2

Relative gene expression analysis of fibroblast (SPDF) after treatment with 50 ng/ml IL-4 or IL-13. The figure illustrates the differential response of SPDFs to cytokine stimulation. Analyzed were POSTN, CCL26, CCL11, CCL2, TNC, and IL-6. Reproducibility varies between PAFs: CCL26 shows consistent results, while CCL2 and IL-6 are less reliable and more difficult to reproduce. Significance is marked: * (p<0.05), ** (p<0.01), *** (p<0.001), **** (p<0.0001). Data are presented as mean ± SD (n = 4).

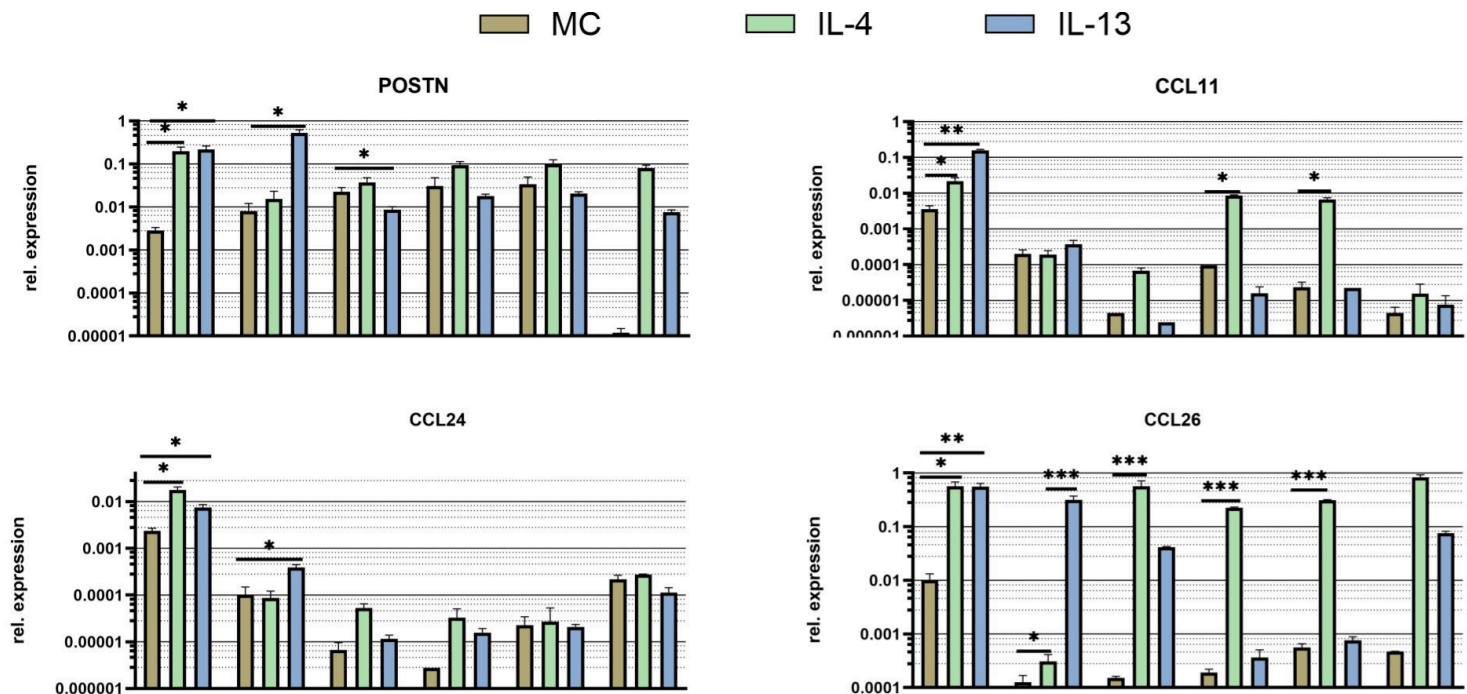


Figure 3

Relative gene expression analysis of epithelial cells (SP) cultured under ALI and submerged conditions after treatment with 100 ng/ml IL-4 or IL-13 (n=6). The figure illustrates the differential response of ALI cultures to the cytokine stimulation. While CCL26 is highly reproducible, the other two factors showed diverging reactions. The PAFs POSTN, CCL11 and CCL24 showed less reproducible results. Significance is marked: * (p<0.05), ** (p<0.01), *** (p<0.001). Data are presented as mean ± SD (n = 6).

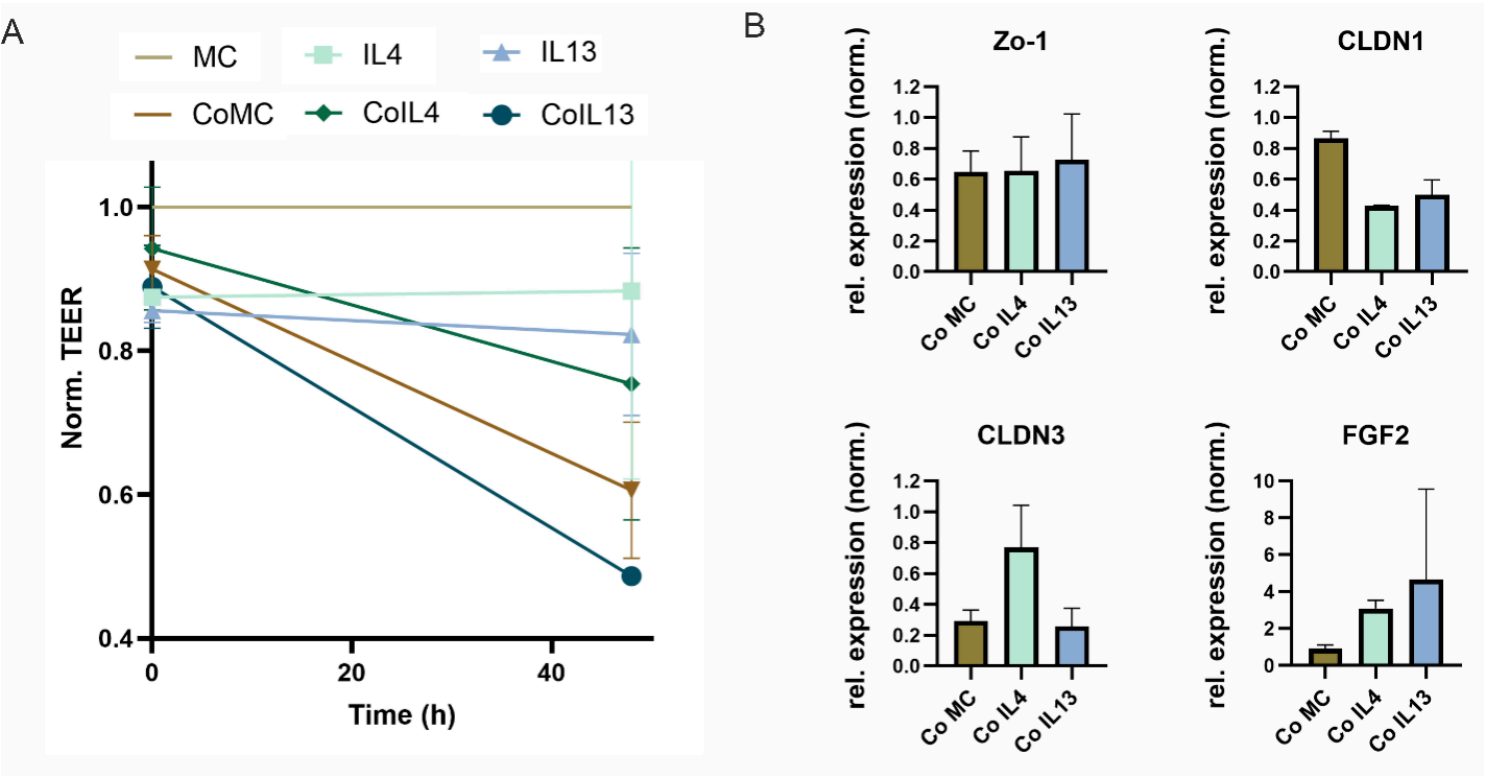


Figure 4

TEER and expression values of epithelial cells from two patients cultured under different conditions for 48 h (n = 2). A Six conditions are shown: monoculture control (brown), monoculture + IL-4 (green), monoculture + IL-13 (blue). The same conditions under co-culture conditions are shown in darker colors. TEER values in co-culture were consistently lower than in monoculture, showing a significantly lower value for IL13-treated co-cultures. B Evaluation of the expression of thigh junction transcripts in epithelium and FGF2 in fibroblasts relative to the monoculture MC. Expression of tight junctions is diversely downregulated in coculture; the expression of FGF2 is particularly upregulated upon treatment with the two cytokines.

Supplementary Files

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