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Physical and immunological barrier of human primary nasal epithelial cells from non-allergic and allergic donors

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ABSTRACT

The epithelial cell-derived cytokine milieu has been discussed as a "master switch" in the development of allergic disease.

To understand the role of innate immune response in nasal epithelial cells during allergic inflammation, we created and established a fast and minimally invasive method to isolate and culture human nasal epithelial cells from clinically and immunologically well characterized patients. Human nasal epithelial cells from non-atopic volunteers and from allergic rhinitis patients were compared in respect to their growth, barrier integrity, pattern recognition, receptor expression, and immune responses to allergens and an array of pathogen-associated molecular patterns and inflammasome activators.

Cells from nasal scrapings were clearly identified as nasal epithelial cells by staining of pan-Cytokeratin, Cytokeratin-14 and Tubulin. Additionally, Mucin 5AC staining revealed the presence of goblet cells, while staining of tight-junction protein Claudin-1, Occludin and ZO-1 showed the ability of the cells to form a tight barrier. Cells of atopic donors grew slower than cells of non-atopic donors. All nasal epithelial cells expressed TLR1-6 and 9, yet the expression of TLR-9 was lower in cells from allergic rhinitis (AR) donors. Additionally, epithelial cells from AR donors responded with a different TLR expression pattern to stimulation with TLR ligands. TLR-3 was the most potent modulator of cytokine and chemokine secretion in all human nasal epithelial cells (HNECs). The secretion of IL-1β, CCL-5, IL-8, IL-18 and IL-33 was elevated in HNECs of AR donors as compared to cells of non-atopic donors. This was observed in the steady-state (IL-18, IL-33) as well as under stimulation with TLR ligands (IL-18, IL-33, CCL-5, IL-8), aqueous pollen extracts (IL-18, IL-33), or the inflammasome activator Nigericin (IL-1β).

In conclusion, nasal epithelial cells of AR donors show altered physical barrier responses in steady-state and in response to allergen stimulation. Cells of AR donors show increased expression of

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pro-inflammatory and IL-1 family cytokines at baseline and under stimulation, which could contribute to a micromilieu which is favorable for Th2.

Keywords: Allergic rhinitis, Nasal epithelium, Pattern recognition receptor, Pollen, Inflammation

INTRODUCTION

The airway mucosal surface is an essential area of signal exchange between the host and its environment. It is becoming increasingly clear, that the airway mucosa does not only constitute a physical barrier to irritants and pathogens but also participates in maintaining the immunological barrier. For instance, airway epithelial cells express receptors for pathogen-associated molecular patterns (PAMPs) and respond to microbial antigens or metabolites by mucus synthesis or production of chemokines, cytokines, and antimicrobial compounds.[1–3]

Allergic rhinitis (AR) is caused by hyperresponsiveness to environmental bioaerosols, such as house-dust mite feces, fungal spores, or plant pollen. AR is characterized by acute, recurrent, or chronic Th2- and IgE-mediated inflammation of the nasal mucosa, involving the activation of resident mast cells and dendritic cells as well as tissue infiltration by immune cells.[4] The nasal epithelium of AR patients is characterized by mucus hypersecretion, edema, goblet cell hyperplasia, tissue damage, remodeling,[5] and impaired barrier function.[6] As the epithelial cell-derived cytokine milieu has been found to be a "master switch" in the development of allergic disease,[7] studying the immunobiology of the nasal epithelium may increase knowledge about the pathomechanism of AR. Sources for primary cell cultures are usually explants from surgeries of normal (turbinectomy) or pathological (polyps, tumors) tissues. Cultures obtained from these sources provide decent results, however, it is difficult to obtain nasal epithelial cells from individuals with defined sensitization profiles. Moreover, material obtained from surgical procedures might not fully mirror physiologic conditions.

The aim of the present study was to optimize a minimally invasive method to isolate and culture HNECs from defined donors and to characterize these cells with respect to physical and immunological barrier functions. We therefore compared HNECs from healthy volunteers and from seasonal allergic rhinitis (SAR) patients sensitized to pollen and assessed the growth, pattern recognition receptor expression, and immune responses to an array of PAMPs and airborne allergens.

METHODS

Cell donors

For nasal curettages, healthy, non-atopic volunteers and volunteers with symptomatic seasonal allergic rhinitis (SAR) and allergic sensitization to pollen were recruited as cell donors after written informed consent. Allergic sensitization against a common aeroallergen panel (tree-, grass- and weed-pollen, fungal spores, house dust mite, cat and dog dander, fungal spores) was determined by serum ImmunoCAP (Thermo Fisher, Massachusetts, US). In addition, tissue specimens were obtained during turbinoplastic surgery of otherwise healthy adult patients with or without SAR. Prior to surgery, patients gave their written informed consent. Allergic sensitization against common aeroallergens was determined by serum ImmunoCAP as described above.

Isolation and expansion of human nasal epithelial cells from nasal curettages

Human nasal epithelial cells (HNECs) were obtained by scraping the surface of the middle meatus bilaterally using a rhino-proR curette (Arlington Scientific, Springville, US). The scraped cells were sterilized using gentamycin (Invitrogen, Carlsbad, US) and 100× Antimycotics-Antibiotics solution (ThermoFisher Scientific, Waltham, MA, USA) and diluted in Minimal Eagle's Medium (MEM; ThermoFisher Scientific). Cells were washed with D-PBS and detached from each other by resuspending in 0.25% Trypsin-EDTA

(ThermoFisher Scientific). Cells were added to Mitomycin C arrested murine 3T3 fibroblasts as feeder cells in medium containing a 1:1 mixture of DMEM (ThermoFisher Scientific) and Ham's F-12 + Nutrient Mixture (ThermoFisher Scientific), 10% fetal calve serum (FCS, HyClone, GE Healthcare, South Logan, US), 1% Penicillin/Streptomycin (ThermoFisher Scientific), and Growth Medium Supplements of Airway Epithelial Cell Growth Medium (PromoCell, Heidelberg, Germany). HNEC-feeder cell co-cultures were incubated at 37 °C, 6.5% CO_2 for at least 4–5 days before the first medium change or first passage. For stimulation experiments, second-passage HNECs were used.

Isolation of HNECs from turbinoplastic surgery specimens

Turbinoplastic surgery specimens were disinfected with gentamycin (ThermoFisher Scientific) and Antimycotics-Antibiotics solution (ThermoFisher Scientific) diluted in MEM, cut into small pieces, rinsed with D-PBS and the HNECs detached by incubation in 0.25% Trypsin-EDTA and passing the tissue through a 30 µm cell strainer. The cells were spun down and half of the cells were stained immediately for flow cytometry while the remaining cells were used for culture as described above.

Reagents and allergen extracts

Pollen grains from birch (*Betula pendula*), timothy grass (*Phleum pratense*), and common ragweed (*Ambrosia artemisiifolia*) were isolated from self-collected male inflorescences by sieving, and aqueous pollen extracts (APEs) were prepared essentially as described in Gilles S et al.[8]. Total protein content was determined by Bradford assay. For stimulation of HNECs, APEs (Bet-APE: birch; Phl-APE: timothy grass; Amb-APE: ragweed) were diluted in Airway Epithelial Cell Growth Medium (Promocell). House dust mite (HDM) extract was purchased at Citeq Biologics, Groningen, The Netherlands; TLR ligands (PolyIC, LPS, CpG-ODN 2006, Pam3Cys, Flagellin) were purchased at InvivoGen, Toulouse, France. Nigericin was from (Sigma Aldrich, St. Louis, Missouri, US).

Stimulation of HNEC monolayer cultures

HNECs were seeded into 24 or 12 well plates, at densities of 5×10^4 cells/ml or 1×10^5 cells/ml, respectively, in complete Airway Epithelial Cell Growth Medium (PromoCell) and grown for 5 days at 37 °C, 5% CO_2. At 80% confluence, cultures were changed to Airway Epithelial Cell Growth Medium without hydrocortisone and treated with either flagellin (100 ng/ml), lipopolysaccharide (LPS; 100 ng/ml), Pam3Cys (1 µg/ml), PolyIC (10 µg/ml), CPG (5 µg/ml), nigericin (10 ng/ml), or APEs (1.0 µg total protein/ml). Unstimulated HNECs served as control. After 24 h IL-1β, CCL-20, CCL-5, CCL-22, IL-33 (R&D Systems, Minneapolis, US) IL-1α, CCL-2, IP-10, GM-CSF, IL-8 (BD Pharmingen, San Jose, US), IL-18 (eBioscience, San Diego, US) and HBD-2 (Peprotech, Hamburg, Germany) were measured in cell culture supernatants by ELISA.

Stimulation of differentiated HNEC cultures

For transepithelial electrical resistance (TER) experiments, HNECs were grown in air liquid interface (ALI) cultures. HNECs were seeded in Transwell® permeable supports (diameter 6.5 mm, polyester membrane with 0.4 µm pores, Corning Life Sciences) at a density of 1.5×10^5 cells in complete Airway Epithelial Cell Growth Medium mixed 1:1 with DMEM (Invitrogen). The medium was changed every other day. After day 5, the medium was aspired from the apical compartment. Medium of the basolateral compartment was changed every 2 days. TER was monitored once a week using a Millicell ERS Volt-Ohm-meter (Merck, Kenilworth, NJ, US). Experiments were carried out 21 days after the air-lift, when the cultures reached a TER higher than 2000 Ω. Therefore, cultures were stimulated apically with Bet-APE (1.0 µg total protein/ml), Phl-APE (1.5 µg total protein/ml), Amb-APE (0.5 µg total protein/ml) or HDM extract (100 µg/ml). EDTA (10 mmol/ml, Invitrogen) was used as positive control for TER decrease, and medium treated wells were used as baseline control. TER was measured prior to stimulation and monitored over time. Measurements were normalized with reference to the values before stimulation ($t = 0$ h) and to the control values.

RT-qPCR analysis

For investigation of TLR mRNA expression in unstimulated HNECs, passage one cells were harvested, and total RNA was isolated, using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. To investigate the TLR expression in TLR-ligand-stimulated HNECs, the cells were stimulated for 6 h, harvested, and total RNA was isolated. RNA concentration and quality were estimated using the BioDrop Touch spectrophotometer (BioDrop, Cambridge, UK). The mRNA was reverse transcribed to cDNA using the iScript™ cDNA Synthesis Kit according to manufacturer's protocol (BioRad, Munich, Germany). The quantitative real-time PCR (RT-qPCR) was performed using iTaq™ Universal SYBR® Green kit (BioRad). Primer sequences are shown in suppl. table 1. The PCR conditions were: 95 °C for 30s, followed by 40 cycles of a 5s denaturation step at 95 °C and a 30s annealing step at 60 °C. A melting curve was performed at the end of each RT-qPCR run by increasing the temperature in a stepwise manner by 0.5 °C every 5s, from 60 °C to 95 °C. For each primer pair, reaction without template cDNA served as a negative control (NTC). Additionally, total RNA was run as template control to check for possible contamination with genomic DNA (data not shown). All RT-qPCR reactions were performed in triplicate with RNA from 2 different sets of samples. Relative transcript levels were calculated by the comparative C(T) method[9] and multiplied with (−1). GAPDH, Tubulin, 18 S, EF1α, SDHA and Keratin-18 were tested for suitable housekeeping controls beforehand (suppl. fig. 1). 18 S showed to be the most suitable, and therefore it served as housekeeping control for expression of TLR and inflammasome genes.

Flow cytometric analysis of HNECs

Surface and cytosolic expression of TLRs and cytosolic expression of TLR adapter proteins in HNECs were analyzed using multicolor flow cytometry. HNECs from conchotomic surgery specimens and from curettages were subjected to flow cytometry directly after isolation. Additionally, HNECs from nasal curettages were stimulated with TLR ligands in 12 well culture plates for 24 h prior to flow cytometry. The cells were pre-incubated with FcR blocking reagent (Miltenyi Biotech, Bergisch Gladbach, Germany) and subsequently stained with saturating concentrations of PE conjugated mouse anti-CD281/TLR1, BV421 conjugated mouse anti-CD282/TLR2, AlexaFluor 700 (AF700) conjugated mouse anti-CD284/TLR4 (eBioscience), AF647 conjugated mouse anti-CD285/TLR5, and FITC conjugated mouse anti-CD286/TLR6 (abcam, Cambridge, UK). For staining of intracellular antigens, cells were fixed and permeabilized using the PerFix-nc kit (Beckman Coulter, Krefeld, Germany) following the manufacturer's instructions, and stained with PE conjugated mouse anti-CD283/TLR3, APC conjugated rat anti-CD289/TLR9 (BD Pharmingen, Heidelberg, Germany), FITC conjugated mouse anti-human TRIF (LifeSpan Bioscience, Seattle, US) and AF405 conjugated mouse anti-human MyD88 (R&D Systems). Non-viable cells, staining positive for PromoFluor840 (PromoKine, Heidelberg, Germany), were excluded from the analysis. For HNECs from conchotomic surgery specimens PerCP conjugated mouse anti-CD45 (BD Pharmingen) was used to exclude leucocytes. FMOs and matched isotype control mouse antibodies were used in control samples. Cells were acquired using a Beckmann Coulter CytoFlex LX flow cytometer equipped with Cytexpert software (Beckman Coulter). Data were analyzed using Kaluza software (Beckman Coulter).

Scanning electron microscopy

To characterize the differentiated HNEC cultures, scanning electron microscopy (SEM) was performed. 14–21 days old ALI cultures of HNECs with a TER of at least 1000Ω were fixed with 2% glutaraldehyde in 1 M cacodylate buffer. Subsequently, cells were dehydrated in 1% OsO_4 in 1 M cacodylate buffer (pH 7.2) for 1 h at 4 °C, and finally washed twice with cacodylate buffer. Samples were then sputtered with 3 nm Au-coating in order to avoid charging effects. Visualization of the sample surface was realized by SEM using a Zeiss Merlin SEM with field emission gun. The SEM was operated with an acceleration voltage of 3 kV and a current of 100 pA. Imaging of the topography was done using an in-lens secondary electron detector.

Immunofluorescence staining

HNECs were seeded into removable 8 well silicone cultivation chambers (ibidi, Planegg, Germany) and grown for 5 days in complete Airway Epithelial Cell Growth Medium (PromoCell) at 37 °C, 5%CO_2. At 80–100% confluence cells were fixed with 4% paraformaldehyde, rinsed, and permeabilized with PBS plus 0.1% Triton X and 0.02% SDS. Subsequently, cells were blocked with 10% goat serum, incubated with either rabbit anti-wide spectrum Cytokeratin (1:200, abcam), mouse anti-Mucin 5AC (1:100, abcam), mouse anti-acetylated alpha Tubulin (1:200, abcam), mouse anti-Cytokeratin 14 (Sigma Aldrich) or rabbit anti-Claudin-1 (1:50, Invitrogen), rabbit anti-ZO-1 (1:50, Invitrogen), mouse anti-Occludin (1:50, Invitrogen) and then incubated with secondary antibodies: goat anti-mouse AF488 (1:2000, Invitrogen), donkey anti-rabbit AF488 (1:2000, Invitrogen) or goat anti-rabbit PE (1:100, Santa Cruz, Dallas, US). Images were recorded on a DMi8 microscope using LAS X Life Science microscope software (Leica, Wetzlar, Germany).

Statistical analysis

We used Mann-Whitney test to compare differences between 2 groups and one-way ANOVA to test for significant differences between several groups. Data of TER measurements were normalized to eliminate high inter-donor variability. To investigate how dependent variables (cytokine release; gene expression) were modified by different stimulation conditions and donor atopy status (categorical predictors) we applied General Linear Models (GLM) and full-factorial ANOVA. To check for significant differences among factors, we applied post-hoc Bonferroni test. Statistical analyses were made using Prism version 6.0 (Graph Pad) or SPSS software (IBM). If not indicated otherwise, data are presented as mean $\pm$ standard error of the mean (SEM). Differences between samples were considered statistically significant if $p < 0.05$.

RESULTS

HNECs from non-atopic donors exhibit faster growth than cells from allergic rhinitis donors

We first compared the growth characteristics of HNECs isolated from non-atopic and SAR donors. HNECs from SAR donors showed a tendency towards slower growth than cells from non-atopic donors (Fig. 1A). Moreover, we observed significantly higher numbers of viable cells, but no difference in dead cell counts, and significantly faster cell growth (days to confluence) in cultures from non-atopic donors as compared to SAR donors when first-passage HNECs were stained with Trypan blue (Fig. 1B).

To determine the effect of the layer of feeder cells co-seeded with the epithelial cells, HNECs obtained from curettages were distributed in 2 wells of a 6-well plate – 1 well with mitomycin-arrested murine 3T3 fibroblasts and 1 well without. Cell growth kinetics of HNECs on fibroblast feeder cells were determined microscopically by assessing the sizes of epithelial cell "nests" developed on the feeder layer over time. We observed that cell growth was only constant in co-cultures with the fibroblasts (suppl. fig. 2).

HNECs from curettages express epithelial cell markers and form goblet cells

We then analyzed the HNEC via different microscopic techniques. Immunofluorescence microscopy of monolayer cultures showed the expression of cytokeratins with a typical pattern for epithelial-type cells and an extensive network of keratin filaments distributed throughout the cytoplasm (Fig. 2A). Approximately 20% of the HNECs expressed Mucin 5AC, a marker of goblet cells, mainly in the cytoplasm but partly also on the plasma membrane (Fig. 2B). Beta-IV-Tubulin, a marker for ciliated cells, was expressed in 60–70% of the cells, with different straining intensity (Fig. 2C). Cultures also stained positive for Claudin-1, ZO-1 and Occludin (Fig. 2D–F). This was most apparent on the sites of cell-cell-contacts, suggesting the presence of intact epithelial tight junctions.

The expression of tight junction genes in HNECs of non-atopic and SAR donors was compared using mRNA levels (Fig. 2G). The relative expression of the MUC5AC and the TJP1 (ZO-1) genes was similar between non-atopic and SAR cells, but HNECs from SAR patients showed a statistically significant reduction in the expression of the CDH1 (E-Cadherin) as well as OCLN (Occludin) genes as compared to HNECs of non-atopic donors.

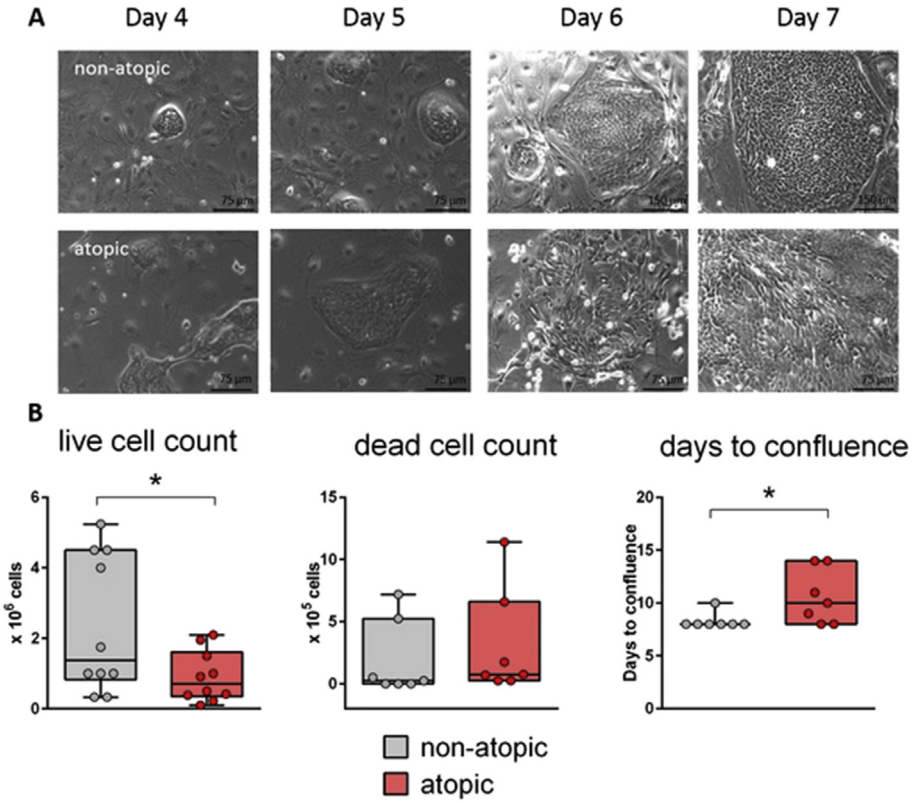

Fig. 1 HNECs from non-atopic donors grow faster than HNECs from SAR donors but have similar survival rates. (A) HNECs obtained from the curettages from non-atopic and AR donors were in a 6 well plate. Cell growth was checked on days 4, 5, 6 and 7 of culture. (B) Time for reaching confluency in cell culture after isolation and cell count at this time point. n = 7 (dead cell count; days to confluence) n = 10 (live cell count). Data are depicted as mean ± min/max. *$p < 0.05$ (Mann-Whitney test).

Scanning electron microscopy of differentiated ALI cultures (Fig. 2H) revealed the presence of large polygonal non-ciliated epithelial-type cells but also polygonal cells with different sizes of microvilli. Additionally, goblet cells with droplets of mucus attached to their surface were observed.

The epithelial integrity is compromised in HNECs from SAR donors

To analyze the physical epithelial barrier, we measured the transepithelial electrical resistance (TER) in ALI cultures of HNECs from non-atopic and SAR patients. HNECs from SAR patients had a significantly lower TER than HNECs from non-atopic donors, starting at 7 days of culture (Fig. 3A). In HNEC cultures from non-atopic donors, TER did not change in response to aero-allergen stimulation (Fig. 3B). In contrast, TER increased in ALI cultures of HNECs from SAR patients after treatment with pollen or HDM extracts and the effect was statistically significant for Phl-APE, Amb-APE and HDM (Fig. 3C).

Atopy status affects innate immune receptor repertoire

To compare the immunological barrier of HNECs derived from non-atopic and SAR donors, we first assessed expression of different TLRs in "steady state", i.e. in unstimulated monolayer cultures. mRNA levels displayed that HNECs expressed the genes for TLR-1-6 and −9, MYD88 and TRIF (suppl. fig. 3). HNECs from SAR donors expressed lower transcript levels of TLR-4 and -9 genes than HNECs from non-atopic donors. TLR-5 and TRIF expression also showed a tendency for lower expression in SAR donors.

Direct *ex vivo* flow cytometry of HNECs from non-atopic and SAR donors was used to assess the protein levels and showed that TLR-1-4, and −9 were expressed (Fig. 4). The expression of intracellular TLRs (TLR-3 and -9) was highest, followed by the extracellular TLR-1, -2 and -4. Only TLR-9 exhibited a significantly decreased expression in HNECs derived from atopic donors as compared to SAR donors. The expression of

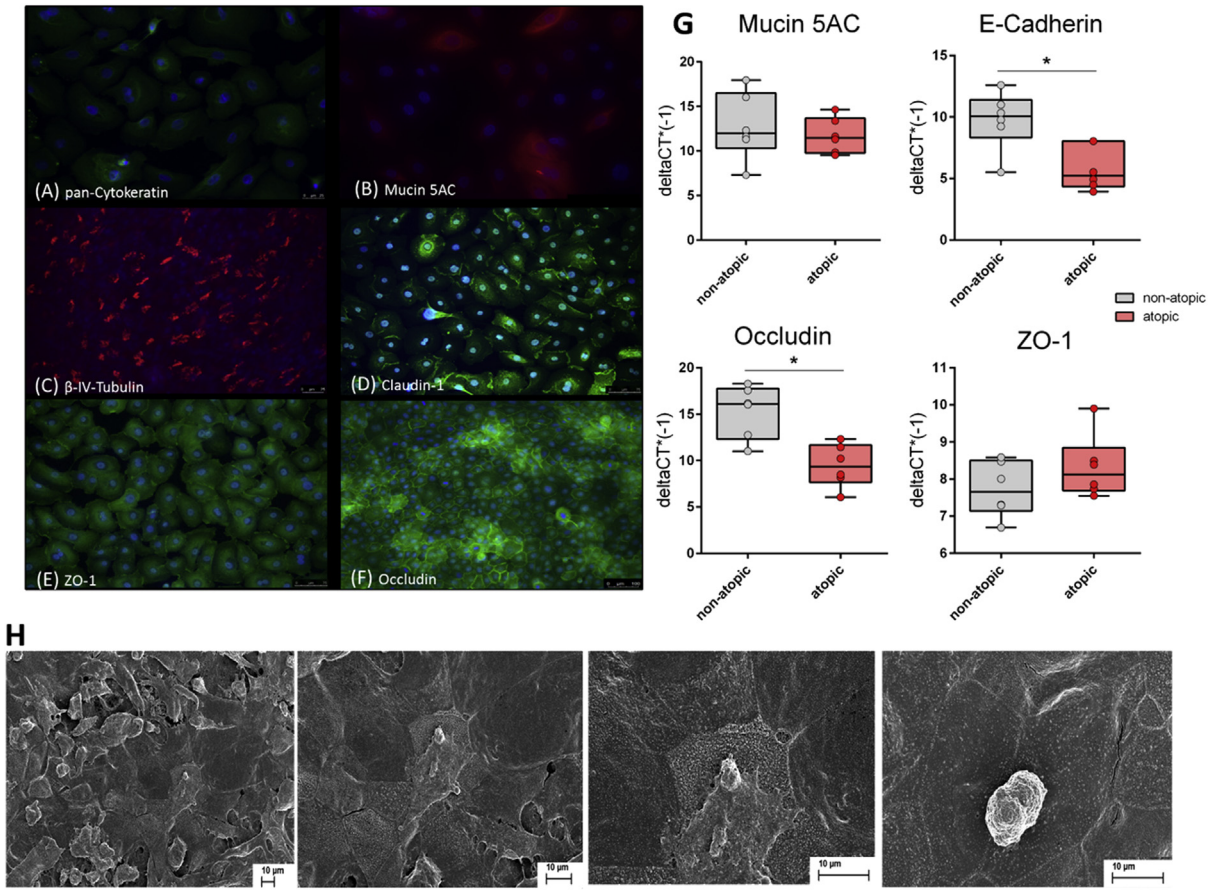

Fig. 2 Cells from nasal curettages were identified as human nasal epithelial cells, form tight junctions and contain goblet cells. Exemplary immunofluorescence staining of HNECs for epithelial cell markers pan-Cytokeratin (A), the goblet cell marker Mucin 5AC (B), the marker for ciliated cells β-IV-Tubulin (C) and tight-junction proteins Claudin-1 (D), ZO-1 (E) and Occludin (F). Nuclei were visualized by staining with DAPI. (G) Relative mRNA expression of Mucin 5AC, E-Cadherin, Occludin and ZO-1 as determined by qPCR in HNECs from non-atopic and AR donors (n = 6 each). *$p < 0.05$ (Mann-Whitney test). (H) Representative scanning electron micrograph of HNEC air-liquid interphase cultures. The cultures consisted mainly of large polygonal non-ciliated epithelial-type cells but also contained polygonal cells with different sizes of microvilli and several goblet cells.

TRIF was, as in the mRNA levels, reduced in HNECs from SAR donors.

HNECs from non-atopic and SAR donors show differential responses to TLR stimulation

Stimulation of HNECs with PolyIC induced the secretion of IL-8, CXCL-10, GM-CSF and CCL-20, whereas CpG induced the secretion of CCL-22. These effects were observed in HNECs of non-atopic and SAR donors alike (suppl. fig. 4). CCL-5 secretion was higher under stimulation with PolyIC, Pam3Cys, Flagellin and CpG stimulation in HNECs of SAR as compared to HNECs of non-atopic donors (Fig. 5A). IL-33 secretion was absent at baseline and after CpG stimulation in HNECs from 2 out of 6 non-atopic donors, resulting in overall significantly higher IL-33 levels in HNECs from SAR patients (Fig. 5B). Secretion of IL-18 was increased in HNECs of SAR patients, both at baseline as well as under all stimulation conditions (Fig. 5C).

HNECs from SAR donors show an altered inflammasome response

The cytokine results prompted us to investigate inflammasome activation of HNECs more closely. We first compared inflammasome-related gene expression on mRNA level, both at baseline and under stimulation with TLR ligands (suppl. fig. 5). HNECs from SAR donors showed significantly lower overall expression of the genes NLRP3, CASP1 and AIM2 ($p < 0.005$), whereas NLRP1 expression was similar in cells of non-atopic and SAR donors. PolyIC stimulation significantly reduced AIM2 gene expression in HNECs from both, non-atopic and SAR donors ($p < 0.05$).

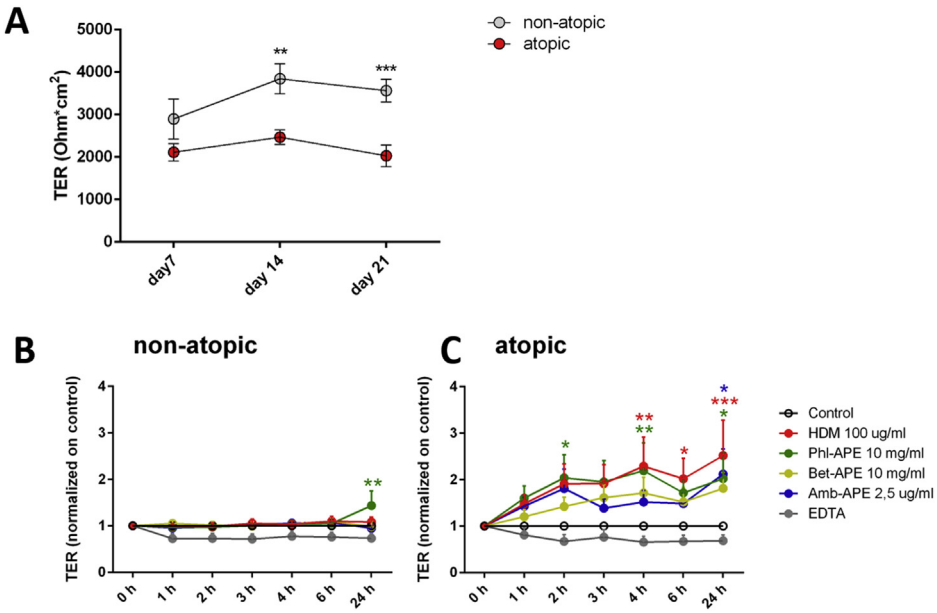

Fig. 3 Transepithelial resistance differs between air-liquid interphase cultures of HNECs derived from non-atopic and SAR donors. (A) Stability of transepithelial resistance (TER) over time in air-liquid interphase HNEC cultures of non-atopic and AR donors (n = 9, each). Data are expressed as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ (B) TER in cultures from non-atopic (n = 11) and (C) AR donors (n = 9) in response to stimulation with different aeroallergens (HDM: house dust mite; APE: aqueous pollen extracts; Bet-APE: aqueous birch pollen extract; Phl-APE: aqueous grass pollen extract; Amb-APE: aqueous ragweed pollen extract). EDTA was used as control to disrupt epithelial integrity. Data are expressed as mean + SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

To assess the inflammasome-triggered cytokine response, we primed the HNECs with TLR ligands (PolyIC, LPS) and subsequently stimulated with the inflammasome activator nigericin (Fig. 6). The overall secretion of IL-33 (Fig. 6A; $p < 0.05$) and IL-18 (Fig. 6B; $p < 0.005$) was significantly elevated in HNECs of SAR donors as compared to cells of non-atopic donors, but stimulation had no significant effect. PolyIC and nigericin specifically induced IL-1β release (Fig. 6C; $p < 0.05$) in cells of 3 of the 6 SAR donors, but not in cells of non-atopic donors. IL-8 secretion was determined as control for TLR ligand stimulation. It was induced under PolyIC stimulation solely in cells of SAR donors (Fig. 6D; $p < 0.001$). Additional stimulation with the inflammasome trigger nigericin did not further enhance the PolyIC-induced secretion of IL-8 (Fig. 6D).

HNECs of SAR donors secrete elevated levels of inflammasome-related cytokines in response to pollen stimulation

Finally we tested whether HNECs from non-atopic and SAR donors show altered inflammasome-related cytokine responses to pollen exposure (Fig. 7). In accordance with the previous results, HNECs of SAR donors produced overall elevated levels of IL-33 (Fig. 7A; $p < 0.01$) and IL-18 (Fig. 7B; $p < 0.05$) as compared to HNECs of non-atopic donors. Overall levels of IL-1β did not differ (Fig. 7C). Stimulation of HNECs with Phl-APE, but not with Bet-APE or Amb-APE significantly induced the release of IL-18 (Fig. 7B; $p < 0.05$) in HNECs of both donor types. Secretion of the inflammasome unrelated chemokine IL-8 did not differ overall between donor types, but was significantly induced by Phl-APE in cells of non-atopic donors (Fig. 7D; $p < 0.05$). In cells of SAR donors, Phl-APE also induced IL-8, however, the induction was not statistically significant (Fig. 7D).

In addition, we also tested for expression of a wider array of other cytokines and chemokines, as well as the antimicrobial peptide HBD2 under pollen stimulation (suppl. fig. 6). HBD2 was induced by Amb-APE mainly in HNECs of SAR patients (suppl. fig. 6A; $p < 0.05$) whereas overall expression of CCL20 (suppl. fig. 6B; $p < 0.01$) and CCL22 (suppl. fig. 6C; $p < 0.05$) was reduced throughout. IL-1α, GM-CSF, CXCL-10 and CCL-2 did not differ between cells of different atopy status, nor were they changed by pollen stimulation (suppl. Fig. 6D–G).

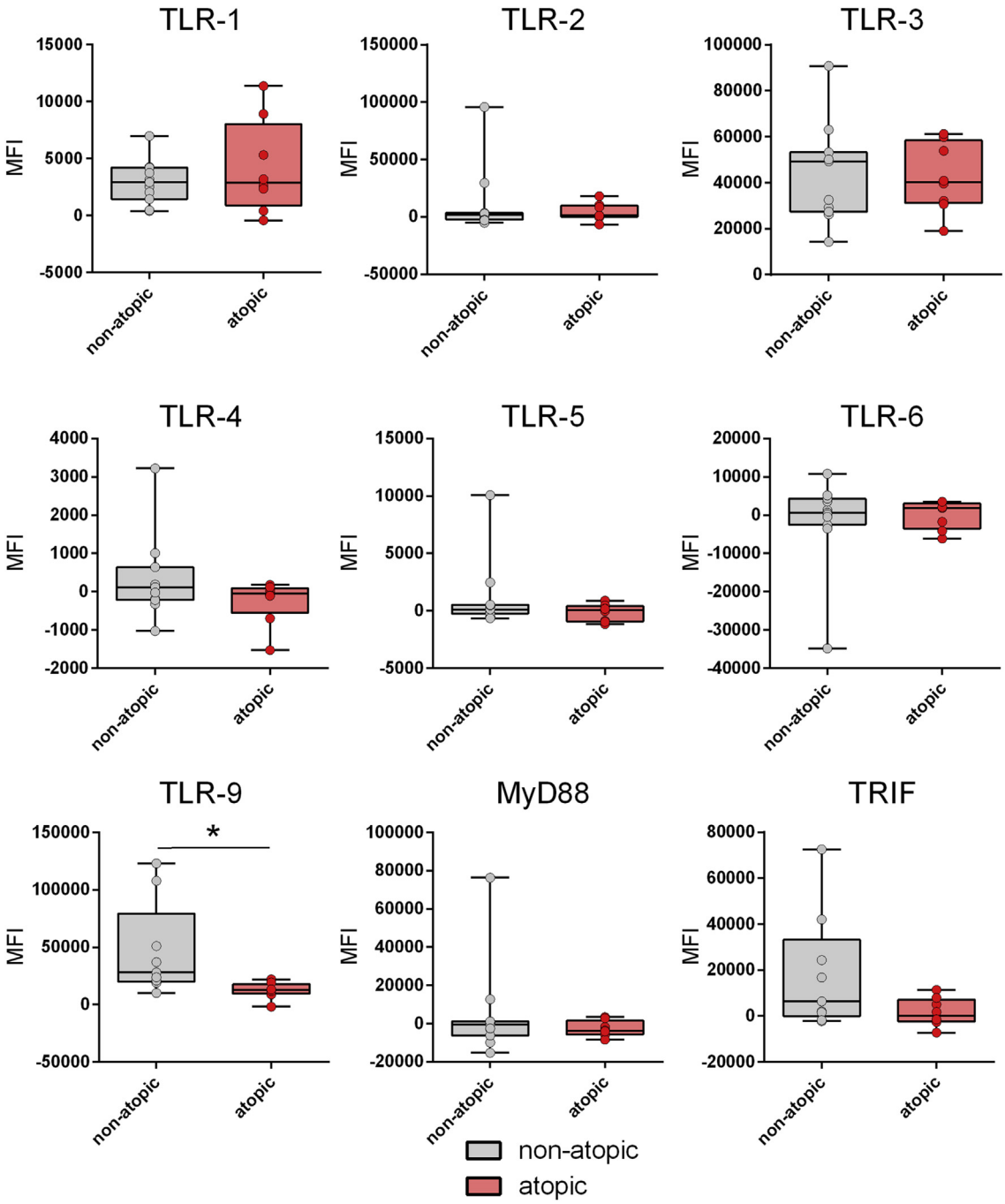

Fig. 4 TLR and TLR adaptor molecule expression differs in HNECs of non-atopic and SAR donors. Direct *ex vivo* TLR and TLR adaptor molecule expression in HNECs of non-atopic (n = 11) and AR donors (n = 8). Directly after isolation, HNECs from curettages and biopsies were stained against TLRs and TLR adaptor molecules and subjected to multi-color flow cytometry. For detection of intracellular proteins, cells were fixed and permeabilized before staining. Expression levels are given as mean fluorescence intensity (MFI). For each marker, the MFI of the respective FMO control was subtracted. $*p < 0.05$ (Mann-Whitney test with Bonferroni multiple testing correction).

DISCUSSION

The ability to use human primary cell culture models in allergy research allows us to harness the potential to compare responses to stimulation between cells derived from donors of different atopy status. Initially, we demonstrate that our method provides sufficient quantities of viable and functional HNECs from non-atopic donors and otherwise healthy SAR patients to perform experiments. Therefore, our expansion protocol could be a valuable tool to improve existing minimal-invasive

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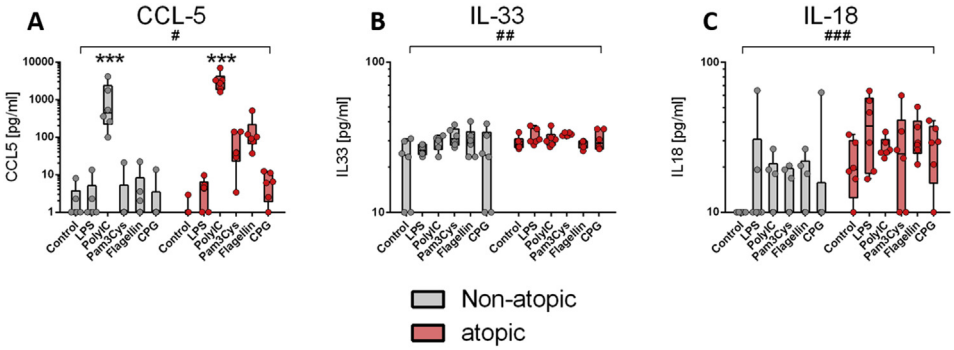

Fig. 5 Patterns of TLR-ligand induced CCL-5, IL-33 and IL-18 release differ in HNECs from non-atopic and SAR donors. (A) CCL-5, (B) IL-33 and (C) IL-18 release of HNECs from non-atopic and AR donors stimulated with different TLR ligands (n = 6, each). Data were analyzed by General Linear Models (GLM) and full-factorial ANOVA with post-hoc Bonferroni test. Cytokine/chemokine levels were the dependent variables, stimulation conditions and donor atopy status the categorical predictors. *: significant difference between stimulation conditions; #: significant difference between cells of different atopy status (non-atopic vs. atopic). ***$p < 0.001$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

HNEC isolation methods. Immunostaining confirmed that monolayer and ALI cultures display an epithelial cell phenotype and contain goblet cells. In a previous study,[10] nasal brushes yielded the highest cell counts and fastest growth rates, whereas the cell viability was similar across the different isolation methods tried. In our study, we obtained comparable cell numbers using the nasal curettage technique and cell numbers expanded very rapidly on feeder cells. A previous study reported diminished levels of E-Cadherin and ZO-1 in nasal epithelium of AR patients.[11] Our results also mirrored these results, and we observed decreased E-Cadherin and Occludin transcript levels in HNECs of SAR when compared to non-atopic donors. Mucin 5AC immunostaining was restricted to 20% of cells in our monolayer cultures designating only a fraction of cells as goblet cells.[12]

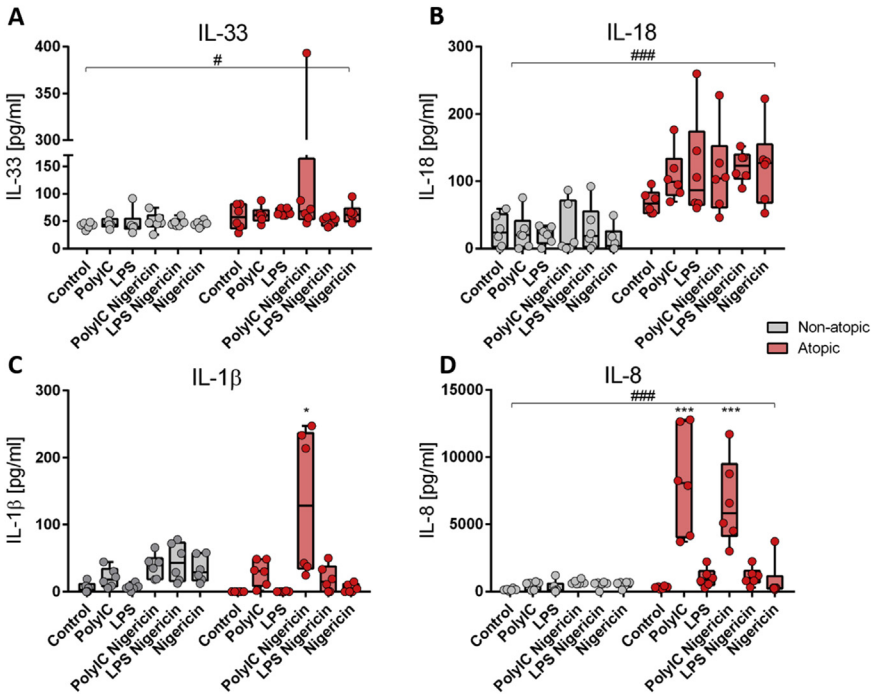

Fig. 6 HNECs from non-atopic and SAR donors differ in their responses to inflammasome stimulation. (A) IL-33, (B) IL-18, (C) IL-1β and (D) IL-8 release of HNECs from non-atopic and AR donors (n = 6, each) stimulated with LPS, PolyIC or with the inflammasome activator Nigericin, with or without prior stimulation with LPS or PolyIC. Data were analyzed by GLM and full-factorial ANOVA with post-hoc Bonferroni test. Dependent variable was cytokine/chemokine release; categorical predictors were stimulation condition and donor atopy status. *: significant difference between stimulation conditions; #: significant difference between cells of different atopy status. *$p < 0.05$; ***$p < 0.001$; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$.

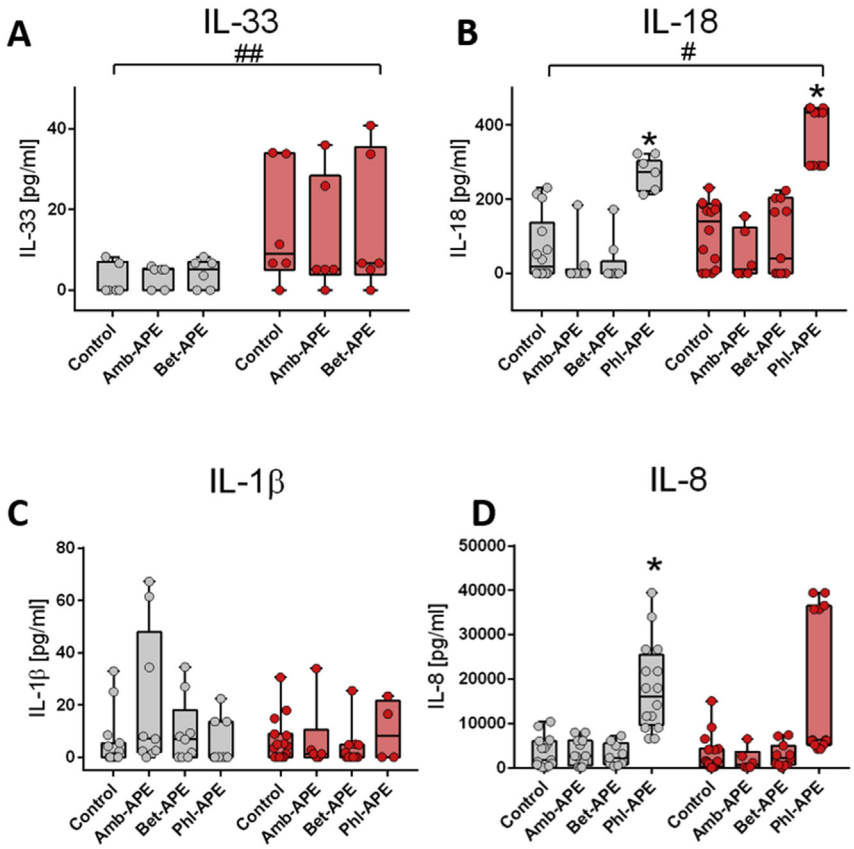

Fig. 7 HNECs of non-atopic and SAR donors show different cytokine responses to stimulation with pollen extracts. Release of (A) IL-33, (B) IL-18, (C) IL-1β and (D) IL-8 in HNECs from non-atopic and AR donors (n = 6–15) stimulated with different aqueous pollen extracts (Bet-APE: aqueous birch pollen extract; Phl-APE: aqueous grass pollen extract; Amb-APE: aqueous ragweed pollen extract). Data were analyzed by GLM and full-factorial ANOVA with post-hoc Bonferroni test. Dependent variable was cytokine/chemokine release; categorical predictors were stimulation condition and donor atopy status. *: significant difference between stimulation conditions; #: significant difference between cells of different atopy status. $^{*}p < 0.05$; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ (ANCOVA with post-hoc Bonferroni test).

Beta-IV- tubulin in ALI cultures was positive only on 60–70% of the cells, suggesting that at least a fraction of the cells have the potential to become ciliated,[13] although we did not observe cilia by SEM.

AR patients have been shown to suffer from increased epithelial barrier permeability.[6,14,15] In line with this, our ALI cultures derived from SAR donors showed a decreased TER at all investigated time points. Interestingly, HDM extract, Phl-APE and Amb-APE were potent inducers of epithelial resistance but only so in ALI cultures derived from SAR donors. A previous study reported similar results in differentiated bronchial epithelial cell cultures exposed to extracts of various pollen types, i.e. timothy grass, ragweed, mugwort, birch and pine.[16] Accordingly, all pollen types induced TER, with timothy grass pollen being the most potent of all pollen types.

Our HNECs expressed TLR-1-4 and TLR-9 on mRNA and protein level, with TLR-3 and TLR-9 most abundant. TLR-4 and -9 mRNA and TLR-9 protein levels were significantly lower in HNECs from SAR donors as compared to HNECs of non-atopic donors. This agrees with a previous study on sinonasal epithelial cells.[17] Likewise, levels of TLR-4 and -9 were shown to be decreased in nasal mucosal biopsies of AR patients as compared to healthy volunteers.[18] These results are of particular interest, as activation of TLR-9 is known to promote Th1-differentiation and antagonize Th2 pathways, an effect which is even more pronounced in non-allergic subjects.[19-21] Apart from reduced expression of TLR-9 we also observed a tendency for a decreased expression of the TLR adaptor molecule TRIF in HNECs derived from SAR donors. TLR-9 ligation by CpG was previously shown to result in physical association of TLR-9, TRIF and TRAF-6 and downstream activation of NF-κB, which, in turn, induced IRF-3 and TGF-β-dependent immune suppressive tryptophan catabolism, possibly protecting against

allergic inflammation.[22] Our results showed that both TLR-9 and TRIF were reduced in SAR as compared to non-atopic HNECs could indicate a down-regulated immune suppressive pathway in SAR nasal epithelium. FACS analysis for TLRs and adaptor proteins was performed immediately after isolation and without prior culture to rule out changes in PRR expression upon prolonged culture (suppl. fig. 7). Several groups investigated TLR expression patterns in nasal biopsies *in situ*, with conflicting results. Past studies found no differences in TLR expression between nasal biopsies from non-atopic and SAR subjects outside the pollen season but increased nasal TLR-3 expression in SAR subjects within the pollen season.[23,24] Intranasal challenge with birch pollen was shown to result in a decrease of TLR-1 and -6 protein expression in nasal biopsies of SAR patients as compared to non-atopic controls.[24] Our study was restricted to HNECs isolated outside the main pollen season and levels of TLR-1, -3 and -6 did not differ between HNECs of non-atopic and SAR donors.

IL-8 was strongly induced in response to timothy grass pollen extract (Phl-APE). In a recent study, repeated nasal allergen challenges with low molecular weight compounds of pollen induced nasal release of IL-8 in SAR patients and even in non-atopic subjects *in vivo*.[25] In a study investigating whole genome transcriptomics of grass pollen treated airway epithelial cells, the most profound changes were related to the genes IL-8, IL-6, IL1A and the transcription factor *fos*.[26] Bronchial epithelial cells were also shown to release IL-8 in response to timothy grass pollen, particularly in response to the pollen-derived flavonoid isorhamnetin**16**.

HNECs of our SAR donors secreted elevated levels of the IL-1 family cytokines, IL-18 and IL-33. In line with our IL-18 results, elevated nasal IL-18 production has been reported in SAR patients outside the pollen season.[27] IL-18 secretion in our HNECs was significantly induced by grass pollen stimulation, irrespective of donor atopy status. This agrees with our own previous data on human primary keratinocytes, which induced IL-18 specifically in response to timothy grass pollen stimulation.[28] Chronic or long-term microbial exposure can go along with elevated tissue IL-33 levels[29] and, in the absence of a Th1 stimulus, might favour Th2 differentiation.[30] For instance, nasal and serum IL-33 levels were found increased in AR patients,[31] and baseline IL-33 mRNA expression correlated with late-phase allergic responses to grass pollen challenges.[32] Moreover, IL-33 was shown to be crucial for the induction of both early- and late-phase responses in HDM- and ragweed-induced murine allergy models.[33,34]

IL-1 family cytokines have been associated with inflammasome activation.[30] HDM, ragweed, and grass pollen extracts were previously reported to activate the inflammasome in human keratinocytes.[28,35] In AR patients, nasal levels of IL-1 family cytokines IL-18 and IL-1α increased parallel to the pollen season and stayed elevated for up to 4 weeks after the last pollen peak, indicating activation of inflammasome related pathways under natural pollen exposure.[27] Accordingly, it was recently reported that the late phase allergic reaction against grass pollen goes along with induction of not only Th2-related cytokine genes but also complement and inflammasome-related genes such as IL-1β.[32] When co-stimulating HNECs from SAR patients with PolyI:C and nigericin we observed a particularly pronounced release of IL-1β in 3 of the 6 patients. Unfortunately, we could not find a common sensitization pattern specifically shared by these patients, except for rather low total IgE-levels, which would be in contrast to previously published data showing elevated IgE and IL-1β serum levels in allergic patients.[36]

In conclusion, our data suggest that HNECs from AR patients may carry a disturbed innate immunity, possibly skewing their innate immune responses away from immune suppression favouring Th2 pathways and finally triggering allergy induction.

Abbreviations

ALI: Air liquid interphase; APE: Aqueous pollen extract; AR: Allergic rhinitis; HDM: House dust mite; HNEC: Human nasal epithelial cell; LPS: Lipopolysaccharide from E. Coli K12 (TLR-4 ligand); MyD88: Myeloid differentiation primary response 88; PAMP: Pathogen-associated molecular pattern; PolyIC: Polyinosinic–polycytidylic acid (TLR-3 ligand); PRR: Pattern recognition receptor; SAR: Seasonal allergic rhinitis; SEM: Scanning electron microscopy; TER: Transepithelial electrical resistance; TLR: Toll-like receptor; TRIF: TIR-domain-containing adapter-inducing interferon-β

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Ethical approval
The ethics committee of Augsburg University Medical School, Germany, approved of the study (ethics statement code: 2016-7).

COI and consent statement
All authors declare no conflict of interest and consent to the publication in its present form.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2020.100109.

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