Influence of nasal polyp tissue on the differentiation and activation of T lymphocytes in a co-culture system

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Abstract. T cell subpopulations in nasal polyps differ from peripheral lymphocytes in patients with chronic rhinosinusitis with nasal polyps (CRSwNP). However, little is known about the modulatory influence of the inflamed nasal polyp epithelial cells on the phenotype of the T cells. The aim of the present study was to assess this interaction. Tissue and blood samples were collected from 16 patients undergoing paranasal sinus surgery. Polypoid tissue was cultured under air-liquid interface conditions. Subsequently, cluster of differentiation (CD)3/CD28 activated peripheral lymphocytes from the same patients were added. After 3 days lymphocytes were separated from co-culture and analyzed by multicolor flow cytometry. Additionally, cytokine expression of the polyp tissue was measured using a human T helper cell (TH)1/TH2/T17 antibody array. Viability staining of CD3+ lymphocytes detected fewer apoptotic cells under co-culture conditions compared with in mono-culture. There was a significantly higher frequency of CD4+ and CD8+ T cells in the co-culture system than in PBMC culture alone. Human leukocyte antigen (HLA)-DR isotype was significantly downregulated on co-cultured CD3+ lymphocytes and CD3+CD4+ T cells compared with the mono-cultured counterparts. Conventional Forkhead box P3+ memory CD4+ T cells and activated regulatory T cells increased in frequency, and resting regulatory T cells decreased in the co-culture. Cytokine analysis identified expression of interleukin (IL)-6, IL-6 receptor, granulocyte-macrophage colony-stimulating factor, transforming growth factor-β and macrophage inflammatory protein-3 in the polyp tissue. In summary, the present study performed a comparison between peripheral lymphocytes cultured with and without nasal polyp tissue cells was performed. The downregulation of HLA and the differentiation of Treg and Tem by nasal polypoid tissue on PBMCs was demonstrated. Interestingly, the in vivo downregulation of HLA-DR on CD3+ lymphocytes, as reported previously, was confirmed in vitro. The inhibitory effect of polypoid tissue on the activation of lymphocytes is a possible pathogenic mechanism underlying CRSwNP.

Introduction

Chronic rhinosinusitis (CRS) is subdivided into two different phenotypes: Chronic rhinosinusitis with nasal polyps (CRSwNP) and without nasal polyps (CRSsNP). The morbidity rate of CRS is relatively high, with 5-15% of the Caucasian population affected (1). However, the etiopathology has remained unclear to date. Recently, study has further investigated the influence of lymphocytes, particularly T cells, and their contribution to maintaining the chronic inflammation of the mucosa. The findings led to a different classification of inflammatory endotypes (2). CRSwNP is characterized by a T helper (TH)2, and CRSsNP by a mixed TH1 and TH17 inflammatory response, though previous data shows heterogeneous cell types in both endotypes with a predominant inflammation type (3). In nasal polyps, the inflammatory process is...
primarily modulated by the expression of interleukin (IL)-4, -5 and -13 (4). Peters et al (5) additionally detected significantly increased levels of IL-6 in CRSwNP, as a further potential pathogenic signaling pathway in this disease. Furthermore, a switch from mainly cluster of differentiation (CD)4+ T cells in peripheral blood to CD8+ T cells in nasal polyps in patients with CRSwNP has been demonstrated (6). Both subpopulations exhibited a significant increase in effector T cells (6). According to the classification of Miyara et al (7), a significant increase in activated regulatory T cells (aTregs) and conventional Forkhead box P3 (Foxp3+) memory T cells (Tconv) was identified in nasal polyps compared with peripheral blood in patients with CRSwNP (6).

Human barrier models are already well established and frequently used for immunological in vitro studies (8-11). Due to their effective illustration of the in vivo situation they are useful for avoiding animal testing (8). Mechanistic papers have depicted the formation of an air-liquid interface model (9), confirmed the similarity of in vitro air-liquid interface models to in vivo models (10) and demonstrated a mucin production in the air-liquid interface equal to normal sputum (11). Certain authors have investigated the influence of cell lines, for instance tumor cells (12) or retinal pigment cells (13), in a co-culture system to determine their phenotype modulation by fibroblasts (12) or T cells (13). Steeplant et al (14) used a co-culture system to measure the epithelial dysfunction in allergic rhinitis. All these studies demonstrate the effectiveness of co-culture systems to assess the influence of one cell type on another cell type.

The question arises if nasal polyp tissue itself is responsible for the consistency of the inflammatory reaction in CRSwNP. The purpose of the current study was to detect the direct influence of the nasal polyp tissue on the differentiation or activation of lymphocytes via the secretion of cytokines. The current study has hypothesized that a major difficulty of T cell investigation in multicolor flow cytometry analysis is the low quantity of these cells within polyp tissue. To circumvent this problem, a co-culture system was developed with nasal polyp tissue and peripheral lymphocytes. Additionally, the cytokine expression of the polyp tissue was measured, since this is responsible for the T cell responses in this system.

Materials and methods

Preparation of human lymphocytes. Heparinized blood samples (10 ml) were obtained intraoperatively by venous puncture from 16 patients (mean age, 51.80±18.12; sex ratio, 6:10 female:male) undergoing paranasal sinus surgery between March 2016 and March 2017. Patients were recruited from the Department of Otorhinolaryngology, Plastic, Aesthetic and Reconstructive Head and Neck Surgery at the University of Würzburg, Würzburg, Germany. Diagnosis of nasal polyposis and indication for surgery was determined according to the European Position Paper on Rhinosinusitis and Nasal Polyps 2012 guidelines (1). Patients with Churg-Strauss syndrome, primary ciliary dyskinesia or cystic fibrosis were excluded. The study was approved by the Ethics Board of the Medical Faculty of Julius-Maximilian-University, Würzburg, Germany (approval no. 16/06), and written informed consent was obtained from all patients.

Peripheral lymphocytes were separated by density-gradient centrifugation for 10 min at 1,000 g at room temperature with 3 ml of Ficoll (Biochrom GmbH, Berlin, Germany), using a membrane-containing 10 ml cell tube (Greiner Bio-One, Kremsmünster, Austria). Tubes were washed twice with phosphate-buffered saline and cell number and viability were determined using a Cell Counter+ Analyzer System (CASY TT; Innovatis Technologies, Inc., Fairfax, VA, USA) according to the manufacturer's protocol. Following centrifugation at 500 x g at 20°C for 5 min, cells were transferred into 1 ml freezing medium, which contained 10 parts fetal bovine serum (Linaris GmbH, Dossenheim, Germany) and one part dimethylsulfoxide. The cell suspension was then stored at -80°C.

Isolation and cultivation of human nasal polypoid tissue cells. All polypoid tissue samples were collected intraoperatively from the same 16 patients undergoing standard paranasal sinus surgery due to CRSwNP as described above. Isolation and cultivation of the cells were performed as previously reported (15,16). Following isolation, the polypoid tissue cells were cultured on porous membrane inserts (Corning Transwell polycarbonate membrane inserts, 0.4 µm pore size, 12 mm diameter; Corning Incorporated, Corning, NY, USA) and covered with 150 µl collagen I (66 ng/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). Culture medium was refreshed every second day. After reaching 70-80% confluence on day 7, the medium apical to the membrane was removed, and nutrition was provided to the cells by adding 1.3 ml Airway Epithelial Cell Medium (PromoCell GmbH, Heidelberg, Germany) per insert under the membrane. At this point, the cultures reached an air-liquid interface condition, which was maintained from day 7 to 14 to stabilize the culture conditions. Subsequently, basal cell medium was removed and a cytokine-free medium was added, which contained 7.5 ml Dulbecco's modified Eagle's medium (DMEM)/F12 and 7.5 µl DMEM-low glucose supplemented with bovine serum albumin (0.4 g albumin/100 ml medium: all from PAA Laboratories, Inc.; GE Healthcare Life Sciences, Little Chalfont, UK), as previously described (12). Peripheral lymphocytes from the same patients were activated with Dynabeads™ Human T-Activator CD3/CD28 (11161D; Gibco™; Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol, and 1x10^6 cells were inserted into the basal compartment. After 3 days in co-culture at 37°C, lymphocytes within the basal compartment were removed and flow cytometry analysis was performed. As a control, peripheral lymphocytes from the same patient were cultured without the Transwell insert in a mono-culture system.

Measurement of cytokine secretion from polypoid tissue under air-liquid interface conditions. Polypoid tissue of 4 patients was cultured according to the method described above, without the peripheral lymphocytes in the basal layers. After 3 days, supernatants were collected and a T_{h1}/T_{h2}/T_{h17} human cytokine array (AAH-TH17-1-2; RayBiotech, Inc., Norcross, GA, USA) with 34 possible targets was performed according to the manufacturer's protocol. Densities of the dot plots were measured by ImageJ v.1.50 (National Institutes of Health, Bethesda, MD, USA).
Fluorescence-activated cell sorting (FACS). The following antibodies were used: Anti-CD45 Pacific Orange (1:300; MHCD4530; Thermo Fisher Scientific, Inc.), anti-CD3 phycoerythrin (PE)-Cyanine (Cy)7 (1:300; 300420) anti-CD4 Pacific Blue (1:50; 300521), anti-CD8a Alexa 700 (1:50; 301028), anti-CD45RA peridinin chlorophyll protein complex-Cy5.5 (1:50; 304122), anti-CCR7 Alexa488 (1:80; 353206), anti-CD4 fluorescein isothiocyanate (1:40; 300506), anti-FoxP3 Pacific Blue (1:25; 320216), anti-human leukocyte antigen (HLA)-DR isotype (AlexaFluor 700; 1:25; 307626), anti-CD52 [also known as cytotoxic T-lymphocyte associated protein 4 (CTLA-4)] PE (1:400; 349906; all from BioLegend, Inc., San Diego, CA, USA) and anti-Ki-67 (1:200; 556027; BD Biosciences, San Jose, CA, USA). Isotype control staining was performed using mouse-immunoglobulin G (IgG) antigen-presenting cell (APC; 1:80; 137214; BioLegend, Inc.) and mouse-IgG PE (1:25; 556027; BD Biosciences). Viability Dye 780 (1:10; 65-0865-14; eBioscience; Thermo Fisher Scientific Inc.) was used to detect apoptotic and dead cells. Following blocking with 25 µg/ml normal mouse IgG (1:50; I5381; Sigma-Aldrich; Merck KGaA) for 15 min on ice, all cells underwent cell surface staining on ice for 30 min, followed by intracellular staining. For intracellular staining of Ki-67, Foxp3 and CTLA-4, cells were treated with 100 µl fixation buffer (eBioscience; Thermo Fisher Scientific, Inc.) per well for 30 min at room temperature. Permeabilization buffer (200 µl) was subsequently applied (eBioscience; Thermo Fisher Scientific, Inc.) followed by staining with anti-Foxp3, anti-CTLA-4 and anti-Ki-67 for 45 min at room temperature. All antibodies were used according to the manufacturer's protocol. FACS analysis was performed using an LSR II flow cytometer and the data were analyzed using FlowJo 10.2 software (FlowJo LLC, Ashland, OR, USA).

Results

Cytokine secretion from polypoid tissue under air-liquid interface conditions. Cytokine secretion of air-liquid interface polypoid tissue cultures was semi-quantitatively measured after 3 days with a T1/T2/T17 antibody array. A secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, IL-6 receptor (IL-6R), macrophage inflammatory protein-3 (MIP-3) and transforming growth factor-β (TGF-β) was identified (Fig. 1).

Viability of lymphocytes under co-culture conditions. Overall, the viability of CD45<sup>+</sup> and CD3<sup>+</sup> lymphocytes was significantly decreased under mono-culture conditions, compared with that of the co-cultured control group (viable cell rate, 52.70±19.42% vs. 66.43±30.83%, respectively; P<0.05; Fig. 2A-C). Furthermore, the percentage of viable CD3<sup>+</sup>CD4<sup>+</sup> (Fig. 2D) and CD3<sup>+</sup>CD8<sup>+</sup> T cells (Fig. 2E) was significantly higher in the co-culture (P=0.02 and P=0.008, respectively; Table I). Between these two subsets, CD4<sup>+</sup>T cells were the dominant subpopulation in both the co-culture and mono-culture (Table I).

No changes in the differentiation of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cell subpopulations. CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cell subpopulations were analyzed via staining of CD45RA and CCR7. The analysis of CD3<sup>+</sup>CD4<sup>+</sup> T cells identified no significant difference in the frequency of CCR7<sup>+</sup>CD45RA<sup>+</sup> naïve, CCR7<sup>+</sup>CD45<sup>-</sup> central memory, CCR7<sup>-</sup>CD45RA<sup>-</sup> effector memory or CCR7<sup>-</sup>CD45RA<sup>-</sup> terminally differentiated T cells (6) between mono- and co-culture (Table I). Effector memory T cells were the major subpopulation in both groups (Table I).

Significant increases in aTreg and Tconv and decrease in resting (r)Treg cells under co-culture conditions. Further determination of CD3<sup>+</sup>CD4<sup>+</sup> T cell subpopulations by staining for...
Foxp3 and CD45RA revealed differences between mono- and co-culture (Fig. 3A). The frequency of Foxp3^CD45RA^- T cells (T_{conv}) (7) was significantly higher in co-culture than among mono-cultured lymphocytes (P=0.01; Table II). The co-cultured lymphocytes also exhibited significantly increased αTregs (CD45RA^Foxp3^{high}CTLA-4^{high}; P=0.04) and significantly decreased βTregs (CD45RA^Foxp3^{low}CTLA-4^{low}; P=0.01) (7) than under mono-cultured conditions. No differences in the frequencies of naïve CD45RA^Foxp3^- and nonsuppressive CD45RA^Foxp3^{low} T_{conv} (7) were determined. In mono- and co-culture, the majority of the CD3^+CD4^+ T cells were naïve CD45RA^Foxp3^- T cells (Table II).

Significant downregulation of HLA-DR on CD3^+ and CD3^+CD4^+ T cells under co-culture conditions. The activation marker HLA-DR (17) was significantly downregulated on
CD3<sup>+</sup>+ lymphocytes (P=0.01) and CD3<sup>+</sup>CD4<sup>+</sup> T cells (Fig. 3B; P=0.0002) on co-cultured compared with mono-cultured lymphocytes (Table III). HLA-DR expression detection was not performed on CD8<sup>+</sup>+ T cells, due to the multicolor flow cytometry panel utilized. Meanwhile, the expression of the intracellular proliferation marker Ki-67 (18) was also measured. No differences in expression were observed among CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells between mono- and co-culture (Table III).

**Table II. Treg subpopulations analyzed by staining for CD4, CD45RA and FoxP3.**

| CD3<sup>+</sup>CD4<sup>+</sup> cell subpopulation | Co-culture | Mono-culture | P-value<sup>a</sup> |
|-----------------------------------------------|-----------|-------------|------------------|
| CD45RA<sup>+</sup>FoxP3<sup>lo</sup>CTLA-4<sup>lo</sup> resting T<sub>regs</sub> | 5.70±2.05 | 11.40±9.63 | 0.01 |
| CD45RA<sup>+</sup>FoxP3<sup>lo</sup>CTLA-4<sup>hi</sup> activated T<sub>regs</sub> | 4.75±2.78 | 3.07±3.05 | 0.04 |
| CD45RA FoxP3<sup>hi</sup> memory cells (T<sub>conv</sub>) | 8.42±3.82 | 7.25±5.40 | 0.50 |
| CD45RA FoxP3<sup>+</sup> memory cells (T<sub>conv</sub>) | 37.93±11.41 | 30.13±14.17 | 0.01 |
| CD45RA<sup>+</sup>FoxP3<sup>+</sup> naïve T cells | 42.40±15.52 | 46.64±17.43 | 0.23 |

Data are presented as the mean ± standard deviation (n=16). *Paired t-test for parametric distribution, Wilcoxon test for non-parametric distribution. CD, cluster of differentiation; T<sub>regs</sub>, regulatory T cells; T<sub>conv</sub>, conventional T cells; FoxP3, Forkhead box P3; CTLA-4, cytotoxic T-lymphocyte.

**Figure 3. Frequencies of CD3<sup>+</sup>CD4<sup>+</sup> T cells in mono- and co-culture. (A) T<sub>regs</sub> subpopulations in co-culture compared with mono-culture. Shown are the frequencies of CD4<sup>+</sup> resting T<sub>regs</sub>, activated T<sub>regs</sub>, FoxP3<sup>hi</sup> memory T cells (T<sub>conv</sub>), FoxP3<sup>+</sup> memory T cells (T<sub>conv</sub>) and naïve T cells. (B) Expression of HLA-DR on CD3<sup>+</sup>CD4<sup>+</sup> T cells in mono- and co-culture. Data are presented as the mean ± standard deviation (n=16). ***P<0.0001; CD, cluster of differentiation; T<sub>reg</sub>, regulatory T cells; FoxP3, Forkhead box P3; HLA-DR, human leukocyte antigen-DR isotype.**

**Table III. Detection of T cells expressing the activation markers HLA-DR and Ki-67.**

| T cell | Co-culture | Mono-culture | P-value<sup>a</sup> |
|--------|------------|-------------|------------------|
| CD3<sup>+</sup> T cells | | | |
| HLA-DR<sup>+</sup> | 7.74±5.06 | 17.42±14.86 | 0.01 |
| Ki-67<sup>+</sup> | 47.30±25.62 | 38.46±26.51 | 0.22 |
| CD4<sup>+</sup> T cells | | | |
| HLA-DR<sup>+</sup> | 8.55±8.05 | 23.10±20.43 | <0.0001 |
| Ki-67<sup>+</sup> | 53.99±25.66 | 55.03±36.25 | 0.88 |
| CD8<sup>+</sup> T cells | | | |
| Ki-67<sup>+</sup> | 62.17±30.52 | 55.93±35.16 | 0.71 |

Data are presented as the mean ± standard deviation (n=16). *Paired t-test for parametric distribution, Wilcoxon test for non-parametric distribution. CD, cluster of differentiation; HLA-DR, human leukocyte antigen-DR isotype.

CD3<sup>+</sup> lymphocytes (P=0.01) and CD3<sup>+</sup>CD4<sup>+</sup> T cells (Fig. 3B; P=0.0002) on co-cultured compared with mono-cultured lymphocytes (Table III). HLA-DR expression detection was not performed on CD8<sup>+</sup> T cells, due to the multicolor flow cytometry panel utilized. Meanwhile, the expression of the intracellular proliferation marker Ki-67 (18) was also measured. No differences in expression were observed among CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> T cells between mono- and co-culture (Table III).

**Discussion**

The current study determined differences in the activation and differentiation of peripheral T cell subpopulations following co-culture with nasal polyp tissue cells. Epithelial cells from nasal polyps were cultured under air-liquid interface conditions and a expression of cytokines, particularly IL-6, IL-6R, GM-CSF, MIP-3 and TGF-β, was measured. This respiratory epithelial cell culture under air-liquid interface conditions is widely used in airway research due to a high functional and morphological in vivo-in vitro correlation (19-21).

Mucociliary differentiation of nasal epithelial cells has been described for regular nasal mucosa (19) and nasal polyps (20). De Borja Callejas et al (21) described a 3D in vitro model for nasal polyposis, which is similar to the air-liquid interface model used in the present study. They also showed a time-dependent mucociliary differentiation of epithelial cells derived from nasal polyps which were cultivated under air-liquid interface conditions. Furthermore, they described
increased levels of pro-inflammatory cytokines such as IL-8 and GM-CSF compared with control nasal mucosa under monolayer culture conditions. Increased GM-CSF levels have been described in vivo in patients with an acute CRSwNP exacerbation (22). Besides GM-CSF, high levels of IL-6 and IL-6R were also detected in the present study. Several studies have highlighted the pro-inflammatory role of IL-6 in patients with CRSwNP and reported on significantly increased tissue levels of IL-6 (5,22) and IL-6R (5). IL-6 and its specific receptor IL-6R appear to play a crucial role in CRSwNP (5), and its signaling pathway is important for T cell recruitment and survival, particularly in preventing apoptosis, preventing the differentiation of Tregs, and in retaining T cells in the local tissue (5,23). Consistent with the presented in vitro study, increased levels of MIP-3 (24,25) and TGF-β (26) have been identified in vivo in patients with CRSwNP. In summary, these findings underscore the reliability of the air-liquid interface model with epithelial cells from nasal polyps and its validity for further immunological studies.

Co-cultured peripheral lymphocytes were significantly more viable and higher frequencies of CD4+ and CD8+ T cells were measured. The secretion of growth factors by the epithelial cells, particularly IL-6, as described above is likely to mediate these findings. This supports the hypothesis that polypoid tissue itself is responsible for maintaining the inflammatory reaction. However, further analysis of T cell subpopulations determined no differences between naïve, central memory, effector memory and terminally differentiated CD4+ and CD8+ T cells compared with mono-cultured peripheral lymphocytes. In mono- and co-culture, effector memory T cells were the major subpopulation among CD4+ and CD8+ T cells, followed by terminally differentiated and naïve cells. The subpopulation with the lowest frequency was central memory T cells in both culture conditions. Previous studies have demonstrated differences in CD4+ and CD8+ T cell subpopulations with differentiation from mostly naïve T cells in peripheral blood lymphocytes to T cells with an effector memory phenotype in lymphocytes from peripheral blood (6,27). Due to the absence of APCs in the in vitro model, cells had to be stimulated by CD3/CD28 prior to adding them to the co-culture. This compromises the direct comparison to in vivo findings, but it appears that polypoid tissue has no immediate influence on the differentiation of T cell subpopulations. Perhaps long-term co-culturing would induce such variations have to be tolerated, and, in addition to this, cells are not immortalized or transformed (31,32). A previous activation of peripheral lymphocytes was necessary in the current study due to the absence of APCs in the co-culture system. This may also influence the results; however, we believe that this fact is likely to be insignificant. The air-liquid interface is able to imitate the effects of cytokines derived from epithelial cells on T lymphocytes, which is considered to be the most important mode of communication (15). However, various settings involving direct contact of lymphocytes with epithelial cells should also be measured. Finally, little is known about the opposite effect, and future studies should focus on the possibility of inducing epithelial cell transformation following co-cultivation with T cells derived from CRSwNP patients.

In conclusion, the present study underscores the influence of nasal polyp epithelial cells in maintaining the inflammatory reaction by expressing pro-inflammatory cytokines and preventing T cells from apoptosis. Furthermore, the study results support that the air-liquid interface co-culture is an
appropriate standardized and reliable model for analyzing the contribution of nasal polyp epithelium cells to the etiopathology of CRSwNP.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

PI was principally responsible for drafting the manuscript and performed, analyzed and interpreted all experiments. AS, NK, RH and CG aided to analyze the data and were major contributors to the writing of the manuscript. SH was involved in the selection, analysis and interpretation of the experiments and was a major contributor to the writing of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Board of the Medical Faculty of Julius-Maximilian-University, Würzburg, Germany (approval no. 16/06). Written informed consent was obtained from each participating patient.

Patient consent for publication

Written informed consent was obtained from all patients for the publication of their associated data.

Competing interests

The authors declare that they have no competing interests.

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