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Selective expansion of human regulatory T cells in nasal polyps, and not adjacent tissue microenvironments, in individual patients exposed to steroids

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ABSTRACT

Severe forms of chronic rhinosinusitis (CRS), a common upper airway inflammatory disorder, are associated with nasal polyps (NPs). NP disease is ameliorated by glucocorticoid (GC) treatment, whose cellular effects are poorly understood. We therefore assessed the influence of GC therapy on NPs in CRS patients, focusing on regulatory T (T_{reg}) cells. T_{reg} cell populations were analyzed by flow cytometry in NPs and control tissues from GC-treated CRS patients and controls. After GC exposure, selective expansion of T_{reg} cells was seen within NPs, and not blood or adjacent ethmoid tissues. To confirm direct GC effects, NPs from the same patients were biopsied prior to, and following, 1 week of oral GC exposure. Direct expansion of Tregs into the same NP bed was detected in 4/4 CRS patients following GC exposure. T_{reg} cell spikes into NPs were secondary to cellular recruitment given limited Ki67 expression within these regulatory cells. Chemokine gene expression profiling identified several chemokines, notably CCL4, induced within NPs upon GC treatment. Neutralization of chemokine receptor/ligand interactions using CCR4 small molecule antagonists reduced T_{reg} migration towards GC-treated NPs in an *ex vivo* migration assay. Our findings suggest that the common use of GCs in the treatment of NP disease leads to recruitment of T_{reg} cells from peripheral sites into NP tissues, which may be critical to the anti-inflammatory effect of GCs. Mechanistically T_{reg} expansion appears to be conferred, in part, by chemokine receptor/ligand interactions induced following corticosteroid therapy.

1. Introduction

A well-developed and orchestrated immunological network can be appreciated within the mucosal surfaces of the upper and lower respiratory airways [1]. In the nasal upper airway, primarily through the use of murine model systems, host-pathogen interactions at the interface between airway and mucosa have become well-defined [1–3]. However, for primates and humans, the complexities of nasal mucosal immunity in the setting of sinonasal diseases, is poorly understood.

Chronic rhinosinusitis (CRS), a prevalent health condition in the US, is an upper airway nasal mucosal disease that affects approximately 1 in 7 adults (31 million patients) annually [4,5]. Patients with CRS present in two major contexts – namely the presence or absence of obstructing nasal polyps (NPs). NPs are inflammatory tissue outgrowths of the upper airway mucosa found in more aggressive and recalcitrant forms of CRS, and patients harboring chronic rhinosinusitis with nasal polyps (CRSwNP) diagnoses demonstrate T_H2-skewed cytokine microenvironment with locoregional eosinophilia [6]. Interestingly, NPs have elevated levels of thymic stromal lymphopoietin and autoantibodies compared to the adjacent sinus tissues, also suggesting a unique NP microenvironment from the surrounding regional upper airway mucosa [6,7].

One of the most effective treatments of CRSwNP disease is the administration of GCs such as prednisone, which suppress elements of the NF-κB and MAPK inflammatory cascade and can diminish

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generalized tissue edema and NP size in the nasal lining [8,9]. Despite the frequent administration of GCs to patients, the precise cellular and molecular effects of steroid use within mucosal microenvironments, including NPs, are unclear. More broadly, GCs are also known to have several actions on lymphocyte and granulocyte subpopulations [10,11], including induction of cell death in circulating T cells [12], and reduction of airway eosinophils, mast cells, T lymphocytes, and dendritic cells by survival pathway inhibition [13]. In concordance with these reports, we have identified that oral prednisone treatment results in a selective decrease in eosinophil numbers within NPs from CRSwNP patients [14]. Alternatively, recruitment of peripheral blood T_{reg} cells, a $CD4^+$ lymphocyte subpopulation, has also been reported upon exposure to GCs in several mucosal and extra-mucosal inflammatory disease states [15]. T_{reg} cell recruitment to non-lymphoid tissues can also be augmented during mucosal inflammation alone, thereby making the effect of steroids on this process unclear [16]. In CRS patients, conflicting reports have shown that the administration of topical intranasal steroids enhances the expression of $CD4^+$ $FOXP3^+$ T_{reg} cells in NPs [17,18], while oral GC therapy reduces Forkhead box P3 $(FOXP3)^+$ T_{reg} cells in NPs [19]. Moreover, TGF-β1, p-Smad2, IL-10, SOCS3, and FOXP3 expression is elevated in steroid-treated NPs compared to controls, suggesting that both cytokines and T_{reg} cells might be involved in the suppression of inflammation [18]. To better address the questions and conflicting findings of the impact of GCs in CRS upper airway mucosal disease, we sought to systematically analyze NP tissues, and the adjacent unaffected human ethmoid sinus mucosa, in patients both before and after GC treatment. As NPs most commonly originate in the ethmoid sinus respiratory mucosal lining, we hypothesized that this experimental design would promote better understanding of the mechanistic basis of possible T_{reg} cell changes within NPs following steroid exposure.

We demonstrate in this report that exposure of CRSwNP patients to systemic steroids causes a direct and significant increase in T_{reg} cell ($CD4^+$ $CD25^{hi}CD127^{lo}$) numbers that is restricted to the NP microenvironment, and not seen in adjacent ethmoid mucosal tissues, or in systemic blood compartment in the same patients. To understand this effect, serial nasal polyp biopsies were taken from the same patients prior to and following GC treatment, revealing a selective spike in $FOXP3^+$ T_{reg} cells, and an alteration in chemokine profiles. These studies provide insights into the immunomodulatory microenvironment within NP tissues, and provide a window to better understanding mucosal immunity of the human nasal upper airway.

2. Materials and methods

2.1. Subjects and specimens

A total of 45 randomly selected patients were included in this year-round study, which was approved by the Institutional Review Boards of both the Stanford University School of Medicine (Protocol ID: 18981, Stanford, CA, USA) and the University of Colorado School of Medicine (COMIRB 11-1442, Aurora, CO, USA). Subjects with confirmed CRS having cardinal symptoms for at least 3 months, inflammatory paranasal mucosal thickening and NPs on endoscopy, and a coronal sinus CT scan demonstrating >3 mm opacification in 2 or more sinuses were included in this study. Informed consent was received from the clinics within the Departments of Otolaryngology-Head and Neck Surgery at Stanford University in Stanford, CA and at the University of Colorado in Aurora, CO. Individual patient data was obtained from questionnaires completed by the attending physician as well as from the available medical records. NP, ethmoid sinus tissue, and blood samples were collected in the outpatient clinic or intraoperatively from patients as specified. Patients with aspirin exacerbated respiratory disease (AERD) were defined clinically based on reported history of ASA/NSAID intolerance with pulmonary reactivity, and confirmed post-operatively by sensitivity to ASA during treatment for ASA desensitization. Subjects had not undergone aspirin desensitization prior to participation in this study.

Patients were excluded if they had a known history of intolerance to corticosteroids, insulin-dependent diabetes, immune deficiency, gastroesophageal reflux disease, glaucoma or ocular hypertension, or an oral steroid-dependent condition. Both patients and controls who used topical corticosteroids, systemic steroids, or leukotriene inhibitors within 3 months were excluded from this study.

Polyp patients (n = 32) were divided into four main cohorts: CRSwNP + prednisone (n = 10), CRSwNP − prednisone (n = 6), AERD + prednisone (n = 8), and AERD − prednisone (n = 8). Controls (n = 9) were also divided into two groups: normal (n = 3) and CRSsNP ± prednisone (n = 3 each) [20,21]. Patients placed on preoperative steroids received oral prednisone at a dose of 30 mg daily for one week prior to surgery. Normal controls (n = 3) were recruited from subjects with nonfunctional macroadenomas undergoing endoscopic transsphenoidal pituitary surgery. The latter controls had no history of CRS or asthma and presented with normal preoperative imaging of the sinuses and normal sinonasal examinations intraoperatively. NPs and ethmoid sinus mucosa were isolated in the CRSwNP and AERD groups; mucosa from the ethmoid sinus alone was isolated in the CRSsNP group, and tissue from the sphenoid rostrum and peripheral regions of this sinus was used in the control group. 8 mL of peripheral blood was collected intraoperatively into heparinized tubes from all patients.

To assess the *in vivo* effects of glucocorticoids on human T_{reg} cell populations in NPs within individual patients, and control for inherent quantitative differences in T_{reg} cells between patients, nasal polyp biopsies were taken from 4 CRSwNP subjects before, and one-week following, a course of oral prednisone (30 mg qD). With each of these four paired samples, flow cytometry, FOXP3 immunohistochemical staining, and RT-PCR were conducted to determine the effects of oral GC treatment within individual human CRSwNP subjects.

2.1.1. Antibodies

Fluorescent conjugated anti-human monoclonal antibodies against CD3, CD4, CD8, CD19, CD45, EpCAM, TCRgd, CD25, CD127, CD39, CD45RA, CD45RO, Ki67, and isotype controls were purchased from BD Biosciences (San Diego, CA). Anti-human monoclonal antibodies against CCR4, CD25, and CD127 (BioLegend, San Diego, CA), GITR and FOXP3 (eBioscience, San Diego, CA) were utilized. The complete list of antibodies used in these studies is presented in Table S1.

2.1.2. High-dimensional 11-color FACS analysis

Peripheral blood mononuclear cells (PBMCs) from all patients were isolated by Ficoll-Hypaque density gradient centrifugation. Cells were then washed twice with phosphate buffered saline (PBS) and used immediately. All nasal tissues from NPs, ethmoid sinus, and sphenoid sinus were washed twice with PBS containing 2 mM EDTA (PBS/EDTA). Tissue samples were mechanically dissociated using microscissors and single cell suspensions were prepared by passing the tissue through a 45 μm nylon mesh filter and collected in RPMI media containing 10% FBS. Red blood cells were removed using red blood cell lysis buffer (BioLegend).

Single cell suspensions obtained from fresh preparations were stained with the aforementioned anti-human fluorochrome-conjugated surface antibodies and isotype controls (Table S1). 1×10^6 cells per sample were stained at room temperature for 25 min, washed in 1640 RPMI media (1600 rpm, 5 min), and subsequently analyzed fresh without fixation. 1 μM propidium iodide was added to all samples prior to data collection to identify dead cells. Intracellular detection of FOXP3 (eBioscience) and Ki67 (BD Biosciences; San Diego, CA) was performed on fixed and permeabilized cells using Cytofix/CytoPerm buffer (eBioscience). Hi-dimensional flow cytometry data were collected on an LSRII FACS instrument (BD Biosciences). FLOWJO (TreeStar, San Carlos, CA) software was used for fluorescence compensation and analysis. Data are depicted as contour plots displaying fluorescence