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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.

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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

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

intensity (FI) axes plotted against cell numbers/FI interval with a total of 256 intervals per parameter.

2.1.3. Immunohistochemistry

Fresh nasal polyps and ethmoid sinus mucosa from CRS patients were formalin-fixed and paraffin-embedded (FFPE) using standard protocols. 4 μM-thick sections from paraffin-embedded tissue blocks were deparaffinized in xylene and hydrated in a series of graded alcohols. Heat-induced antigen retrieval was carried out by pressure cooker pretreatment (121 °C) in Tris/EDTA buffer (pH 9.0 for 10 min). Slides were cooled at room temperature for 30 min, rinsed in PBS and incubated with peroxidase block (DAKO Corporation, Carpinteria, CA) for 10 min. Slides were stained with anti-human FOXP3 antibody (clone: 236A/E7) at a dilution of 1:20 for 2 h at room temperature. Detection was carried out using the DAKO Envision method (DAKO Corporation). Sections were counterstained with hematoxylin and subsequently dehydrated in alcohol and xylene before mounting. Microscopic images were obtained using both a Zeiss Axio Imager 2 microscope (Carl Zeiss Vision, Germany) and an Eclipse E800M microscope with confocal attachment (Nikon, Melville, NY). Microscopy images were captured using a SPOT RT digital camera and corresponding acquisition software (Diagnostic Instruments, Sterling Heights, MI) and acquired with a final magnification of 400 (objective, 40×). FOXP3 nuclear staining was quantitatively scored by two observers in a blinded fashion. Images were processed with Photoshop software (Adobe Systems, San Jose, CA).

2.1.4. Lymphocyte proliferation/T_{reg} cell suppression assays

Ethmoid sinus and nasal polyp tissues derived from the same CRSwNP patients were sampled in the absence of steroids, and following a course of preoperative steroids (6–9 days). PBMCs and single cell suspensions of the ethmoid sinus and nasal polyp tissues were incubated with 1 μM CFSE (Invitrogen, Carlsbad, CA) for 7 min in serum-free media at room temperature. Labeled cells were then washed twice with complete RPMI-1640 medium containing 10% FBS and plated in round bottom 96-well plates at a density of 0.5×10^6/well. For induction of proliferation, cells were incubated with anti-CD3/CD28 conjugated beads (Invitrogen) at beads-to-cell ratio of 1:1. Control cells were left unstimulated. After incubation for 96 h, cells were washed once with PBS, stained with antibodies against CD3, CD4, CD127 and CD25 and analyzed by flow cytometry. Cell divisions were quantified by presence or loss of CFSE peaking fluorescence using FLOWJO software.

2.1.5. RNA isolation

Total RNA from nasal polyps was extracted with TRIzol reagent (Invitrogen) following the manufacturer's instructions. The quantity of total RNA was measured using a NanoDrop ND 1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). 260/280 ratios for all samples were >1.95.

2.1.6. PCR array

For cDNA synthesis, 800 ng of total RNA from each tissue source was transcribed with cDNA transcription reagents using the RT^2 First Strand Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocols. The RT^2 Profiler PCR Array (QIAGEN) for Human Chemokines/Receptors was used to analyze pathway expression variance. The prepared cDNAs were amplified using RT^2 SYBR Green qPCR Mastermix (QIAGEN) according to the manufacturer's recommendations. Gene expression was measured in real-time with an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The following conditions were used for amplification: 1 cycle of 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing at 60 °C for 1 min. A melt curve analysis was performed to verify PCR specificity. The expression level of a gene in a given sample was represented as $2^{-\Delta\Delta Ct}$ where $\Delta\Delta CT = [\Delta CT_{(experimental)}] - [\Delta CT_{(control)}]$ and $\Delta CT = [CT_{(experimental)}] - [CT_{(housekeeping)}]$, where expression levels from GC-

treated NPs were compared to untreated NPs. Data are presented as normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) expression. The data for biological duplicates were analyzed using the SABiosciences PCR Array Data Analysis Software.

2.1.7. Real-time PCR analysis

For cDNA synthesis, 800 ng of total RNA from each tissue source was transcribed with cDNA transcription reagents using the RT^2 First Strand Kit according to the manufacturer's protocols. CCL4, CCR4, CXCL1, CXCL2, and CXCR1 primers (QIAGEN) were used to quantify gene expression. The prepared cDNAs were amplified using RT^2 SYBR Green qPCR Mastermix according to the recommendations of the manufacturer. Gene expression was measured in real-time with an ABI 7900HT Fast Real-Time PCR System. The following conditions were used for amplification: 1 cycle of 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing at 60 °C for 1 min. A melt curve analysis was performed to verify PCR specificity. The expression level of a gene in a given sample was represented as $2^{-\Delta Ct}$ where $\Delta CT = [CT_{(experimental)}] - [CT_{(housekeeping)}]$, where expression levels from GC-treated NPs were compared to untreated NPs. Data are presented as normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) expression.

2.1.8. Protein isolation

Total protein from nasal polyps was extracted with T-PER Tissue Protein Extraction reagent (Thermo Fisher Scientific, Waltham, MA) and Halt Protease Inhibitor Cocktail, EDTA-Free (Thermo Fisher Scientific) following the manufacturer's instructions. Using a polyp tissue to T-PER reagent ratio of 1 g:20 mL, approximately 30 mg of fresh nasal polyps and 600 μL of T-PER reagent were used to isolate total protein. Samples were centrifuged (1600 rpm, 5 min) to pellet cell/tissue debris and the supernatants collected. The quantity of total protein was measured using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Protein was measured using the manufacturer's standard protocol and read at 562 nm.

2.1.9. CCL4 ELISA

400 μg of total nasal polyp protein (before and after steroid exposure) was used for the CCL4 ELISA assay for each sample. The amount of CCL4 protein was determined by the Human CCL4/MIP-1 beta Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's protocols.

2.1.10. Chemotaxis assay

The chemokine-dependent migration of PBMCs was measured using an in vitro 2-chamber migration assay [22]. PBMCs were pre-incubated in a humidified atmosphere containing 5% CO_2 at 37 °C for 12 h prior to doing the chemotaxis assay. Specific migration of T_{reg} cells was evaluated using 5 μm pore polycarbonate filters in a Transwell system (Costar, Cambridge, MA). PBMCs (1×10^6) were suspended in 100 μL migration buffer (RPMI 1640 containing 10% FCS) and added to the upper chamber. A total of 600 μL migration buffer supplemented with 500 ng/mL CCL4 (R&D Systems) was added to the lower chamber. The plates were then incubated for 3 h at 37 °C. Input cells and transmigrated cells were centrifuged and cells were stained with anti-CD3, -CD4, -CD127 and -CD25 antibodies analyzed by flow cytometry. To calculate the chemotactic index, the number of cells migrated in response to CCL4 was divided by the number of spontaneously migrated cells.

2.1.11. Inhibition of T_{reg} chemotaxis to steroid-treated NPs using CCR4 antagonism

Using the same chamber setup for the PBMC chemokine-dependent migration assays, chemotaxis assays using nasal polyps and a CCR4 antagonist were established. PBMCs from the same CRSwNP patients were labeled with 1 μM CFSE (Invitrogen, Carlsbad, CA) and 1×10^6 cells were added to the upper chamber in 100 μL migration buffer (RPMI 1640

containing 10% FCS). A total of 600 μL migration buffer supplemented with 500 ng/mL CCL4, migration buffer with 10 nM CCR4 antagonist (Cat #227013-5MG; EMD Millipore, Billerica, MA), or migration buffer supplemented with 200 mg fresh, steroid-treated nasal polyps was added to the lower chamber (Fig. 5C). Titration assays of the CCR4 antagonist were performed with 1, 10, and 100 nM concentrations, and 10 nM was found to be the optimal concentration. The control used for the CCR4 antagonist was the vehicle. The plates were then incubated for 3 h at 37 °C. Transmigrated cells were centrifuged and cells were stained with anti-CD3, -CD4, -CD127 and -CD25 antibodies and washed again and analyzed by flow cytometry. Migrated CFSE-labeled PBMCs were gated on and T_{reg} cells were quantified based on CD127 and CD25 surface marker expression. Control migration experiments with fresh non-steroid treated NPs were performed prior due to sample availability. To calculate the chemotactic index, the number of cells migrated in response to either CCL4, CCR4 antagonist, or steroid-treated nasal polyps was divided by the number of spontaneously migrated cells.

2.1.12. Statistical analysis

All statistical procedures were performed with Prism 5 Software (GraphPad Inc, San Diego, CA). *P*-values were determined by applying Student's two-tailed *t*-test for independent samples assuming equal variances on all experimental data sets. *P*-values are indicated throughout with * for $P < 0.05$, ** for $P < 0.01$, and *** for $P < 0.001$.

3. Results

3.1. Increased T_{reg} cells in nasal polyps upon administration of oral GCs

One of the fundamental features of CRSwNP is the formation of nasal polyps from the ethmoid sinus mucosal surfaces. Although this human inflammatory disease has been characterized by an abundance of T_H2 cytokines and gene microarray analysis has implicated a down-regulation of Th17 markers in nasal polyps [23,19,24], underlying differences between NPs and the adjacent ethmoid sinus mucosa (the source of NP disease) are poorly defined. To address this, we initially performed phenotypic analyses of resident T cell subsets simultaneously from three tissue subsites – peripheral blood mononuclear cells (PBMCs), adjacent ethmoid sinus mucosa, and NPs – from individual CRSwNP patients (Fig. 1A). $CD3^+$ cells were initially discriminated between αβ and γδ T cells based on the presence of respective TCR chains (Fig. 1B). TCR-αβ cells were further resolved for CD4 and CD8 subsets, revealing no relative differences in $CD4^+$ cell numbers between ethmoid sinus mucosa and NPs. Among CD4 subsets, T_{reg} cells were additionally defined based on CD25 and CD127 expression, with no differences in $CD25^{hi}CD127^{lo}$ T_{reg} cells again seen between mucosal subsites (Fig. 1B). 97–99% of nasal and systemic T_{reg} cells were confirmed to be $FOXP3^+$ by intracellular staining and FACS (Fig. S3). In these studies, PBMCs serve as an important normal control tissue source and control for establishing FACS gating between individual patients.

However, upon administration of a one-week course of oral prednisone therapy to CRSwNP patients for disease treatment, the same phenotypic analyses of T cell subsets indicate that GCs confer selective effects on NPs, in parallel to what is noted clinically. In line with the expected suppressive effects on T cells by GC exposure, an expected reduction in the proportion of γδ T cells in both ethmoid sinus mucosa and NPs was noted (Fig. 1C) relative to the same tissue sites from untreated patients (Fig. 1B). Reference values from normal control samples of PBMC and ethmoid tissues can be found in Table S3. By contrast, T_{reg} cells displayed a statistically significant and selective increase within NPs following GC treatment in CRSwNP patients (Fig. 1C). In an additional subset of aspirin exacerbated respiratory disease (AERD) patients, who present with a more aggressive form of nasal polyposis [25], an overlapping expansion in T_{reg} cells was observed within NPs with no

associated significant cellular changes in the adjacent ethmoid sinus mucosa and peripheral blood following prednisone treatment (Fig. S1).

An analysis of all CRSwNP patients confirmed no significant change in $CD4^+$ lymphocyte levels after GC therapy within NPs (Fig. 1D). Among $CD3^+CD4^+$ T cells, however, a statistically significant increase in T_{reg} cells was observed (Fig. 1E), suggesting a selective mechanism for T_{reg} cell expansion in NP mucosal tissues conferred by exposure to GCs, rather than global cellular density changes. To investigate whether $FOXP3^+$ T_{reg} cells might be increased in NPs following steroid treatment [26], intracellular FOXP3 staining was performed on single cell suspensions of NPs. To exclude activated effector T cells, all $CD4^+$ $CD25^{hi}CD127^{lo}$ T_{reg} cells from both untreated and GC-treated NP samples were found to express the marker FOXP3 (Fig. 1F). Together, these data suggest that $FOXP3^+$ T_{reg} cells in NPs are selectively increased within NPs, but not adjacent sinonasal mucosal tissues, in patients following GC treatment, and confirm and extend findings from earlier work [17,18].

3.2. T_{reg} cells phenotype shifts in steroid-treated NPs

Compared to steroid naïve patients, we found that CCR4 surface expression nearly doubled, and glucocorticoid-induced TNF receptor (GITR) levels notably increased, following GC therapy on T_{reg} cells within NPs (Fig. 2A). Subset analysis using CD45RO, CD45RA, and CD39 surface expression to determine activation state revealed that T_{reg} cells display a predominantly naïve phenotype in nasal polyps when devoid of steroid. Following GC therapy, T_{reg} cells harbored an activated, memory ($CD45RA^-$ $CD39^+$) phenotype (Fig. 2B). Interestingly, in the adjacent ethmoid sinus mucosa, the opposite phenotype was observed, where an increase in naïve ($CD45RA^+$ $CD39^-$) T_{reg} cells was localized to this mucosal subsite (Fig. S2). The skewed presence of an activated T_{reg} cell phenotype within GC-exposed, NP tissues suggests that these cells may provide a direct immunomodulatory role within NPs. Previous studies have shown that corticosteroid therapy can fine tune the capacity of T_{reg} cells to suppress aspects of autoimmune diseases [27,28].

3.3. T_{reg} cell expansion is independent of in situ proliferation

Elevated numbers of activated, T_{reg} cells isolated from NP mucosal tissues following GC therapy raised mechanistic questions regarding T cell recruitment versus proliferation in CRS tissues. The expression of intranuclear Ki67, a proliferation-associated nuclear protein, did not increase following prednisone treatment, thus excluding the possibility of local proliferation in T_{reg} cells within NPs following GC administration (Fig. 2C).

3.4. T_{reg} cells suppress T cell proliferation in steroid-treated NPs

To determine the functional capacity of T_{reg} cells within CRS patients, NPs and adjacent sinus cell suspensions were analyzed for their proliferative capacity following *in vitro* stimulation. Total cell suspensions from both NPs and ethmoid sinus were labeled with CFSE, stimulated over 4 days with anti-CD3/CD28 conjugated beads, and analyzed for cell division by flow cytometry. This potent stimulation signal resulted in an expected increase in proliferation of $CD3^+CD4^+$ T cells at both tissue sites (Fig. S4). Upon exposure to GCs, proliferation of $CD3^+CD4^+$ T cells was profoundly reduced only within the NP group (Fig. S4A). This effect was not restricted to $CD3^+CD4^+$ T cells, as exposure to prednisone in NPs also suppressed $CD3^+CD8^+$ T cell proliferation selectively in NP tissues (Fig. S4B). Taken together, these data suggest that functional T_{reg} cell activities can be modified depending on the pro- and anti-inflammatory contexts found within the NP microenvironment.
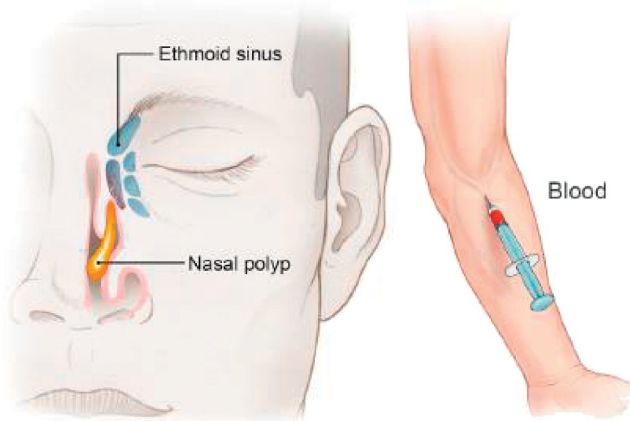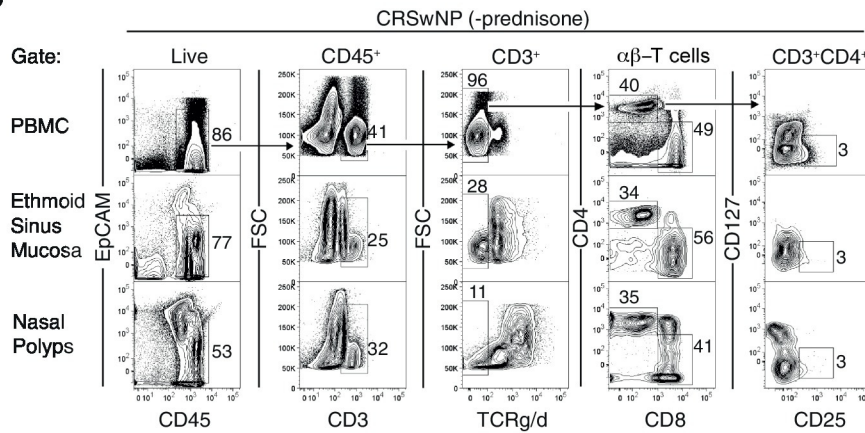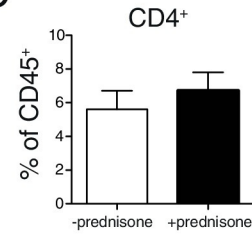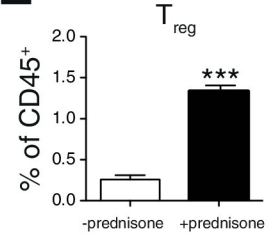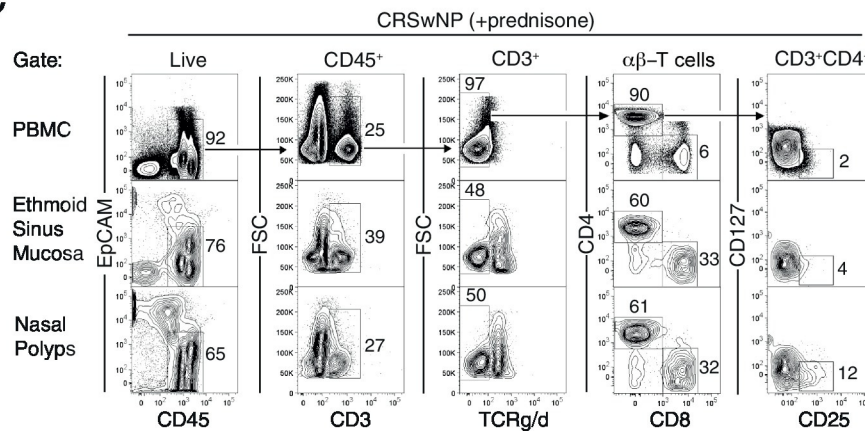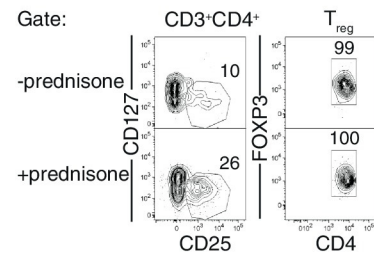
Fig. 1. Phenotypic analyses of T cell subsets from CRS patients reveal a selective increase in T_{reg} cells (CD4$^+$CD25^{hi}CD127^{lo}) in NPs following GC administration. A. Schematic of tissue sites (PBMCs, adjacent ethmoid sinus mucosa, and NPs) that were harvested from human CRSwNP subject. B. Single cell preparations of PBMCs, adjacent ethmoid sinus mucosa, and NPs from CRSwNP patients without steroid exposure were stained with fluorochrome-conjugated antibodies. Top panel shows the analysis from peripheral blood; middle panel shows adjacent ethmoid sinus mucosa; bottom panel depicts NPs. Respective gates are indicated above each column. Leukocytes were initially gated for CD45 expression, with T cells identified based on CD3 expression. CD3$^+$ cells were subsequently resolved for αβ and γδ T cells based on respective TCR chains. TCR-αβ cells were further analyzed for CD4 and CD8 subsets, and regulatory T (T_{reg}) cells were then resolved among CD4$^+$ lymphocytes based on CD25 and CD127 expression, where T_{reg} cells were defined as CD25^{hi}CD127^{lo}. Arrows indicate this progressive gating strategy. Note that nasal tissues have a high proportion of γδ T cells, characteristic of mucosal tissues (middle column panels). Percentages of T_{reg} cells remained constant between NPs and ethmoid sinus mucosa (far right panels). One representative distribution from an analysis of 6 patients is shown. C. Similar analysis panels and gating strategy shown in (A), but now from CRS patients treated for 7 days with GCs. Note that steroid administration results in a decrease in the percentages of γδ T cells in both ethmoid sinus mucosa and NPs (middle column panels) compared to untreated patients (A). There is a 4-fold increase in T_{reg} cells selectively in NPs (far right panels) compared to (A). One representative distribution from an analysis of 10 patients is shown here. D. Quantification of CD4$^+$ cells within NPs before and after GC administration is illustrated in the bar graph. No significant change in CD4$^+$ levels was observed within NP tissues in treated CRS patients (n = 10) compared to untreated patients (n = 6). Error bars represent SEM. E. T_{reg} cell counts in NPs are significantly increased following GC treatment in CRSwNP patients (n = 10) compared to untreated patients (n = 6). Error bars represent SEM. ***, P < 0.001. F. Representative FACS plot confirms augmented numbers of FOXP3$^+$ T_{reg} cells (CD4$^+$CD25^{hi}CD127^{lo}) in NPs. Data depicted are representative of three independent experiments.

3.5. When exposed to GCs, NPs from the same individual patient show a selective expansion of T_{reg} cells

To test whether the selective localization of T_{reg} cells into GC-treated NPs was secondary to recruitment via local chemokine gradients, NP biopsies from the same individual patients pre- and post-GC treatment were examined using flow cytometry, immunohistochemistry, and gene expression analyses to eliminate disparities in patient sampling or cognate differences between patients (Fig. 3A). Using CD25 and CD127 expression for initial gating strategies, we first confirmed that NPs from the same individual afflicted with CRSwNP disease and undergoing GC treatment, exhibited overlapping trends in T_{reg} cells as was observed in our larger patient cohort (Fig. 3B). Immunohistochemical staining of NP tissues confirmed expansion in FOXP3$^+$ cells following prednisone treatment within individual patients (Fig. 3C). Among all samples, there was a statistically significant increase in FOXP3$^+$ cells
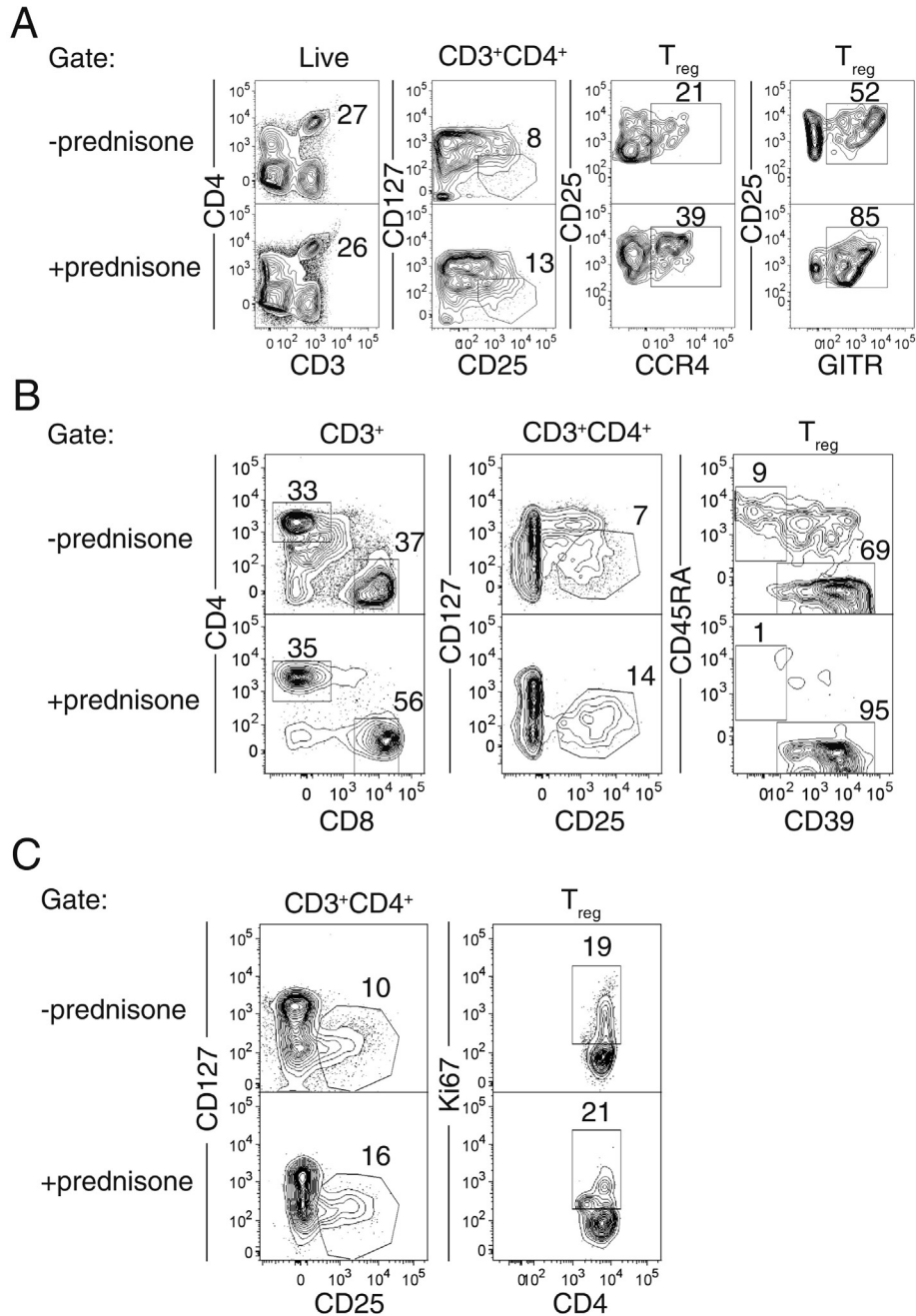

Fig. 2. GC therapy increases the number of activated T_{reg} cells within NPs. A. Cells prepared from untreated and GC-treated CRS nasal polyps were analyzed for distinct T_{reg} cell subsets. CD4$^+$ T cells were resolved based on CD25 and CD127 expression, and T_{reg} cells (CD4$^+$ CD25^{hi}CD127^{lo}) were subsequently gated based on C-C chemokine receptor type 4 (CCR4) and glucocorticoid-induced TNFR-related protein (GITR) expression. Subset analysis of these T_{reg} cells revealed augmented T_{reg} cell surface markers CCR4 and GITR expression, following GC administration (right two panels). B. T_{reg} cells were subsequently gated based on CD45RA and CD39 expression. As in Fig. 1, an increase in T_{reg} cells was seen in NPs following GC treatment. In untreated NPs, subset analysis of T_{reg} cells revealed a primarily naïve phenotype. Following GC therapy, a marked rise in memory, activated (CD45RA$^-$ CD39$^+$) T_{reg} cells was seen in NPs (far right panels). In A and B, data depicted are representative of three independent experiments. C. Following GC treatment, T_{reg} cells from NPs are not stimulated to proliferate based on absence of shift in intracellular Ki67 staining. Data depicted are representative of three independent experiments.
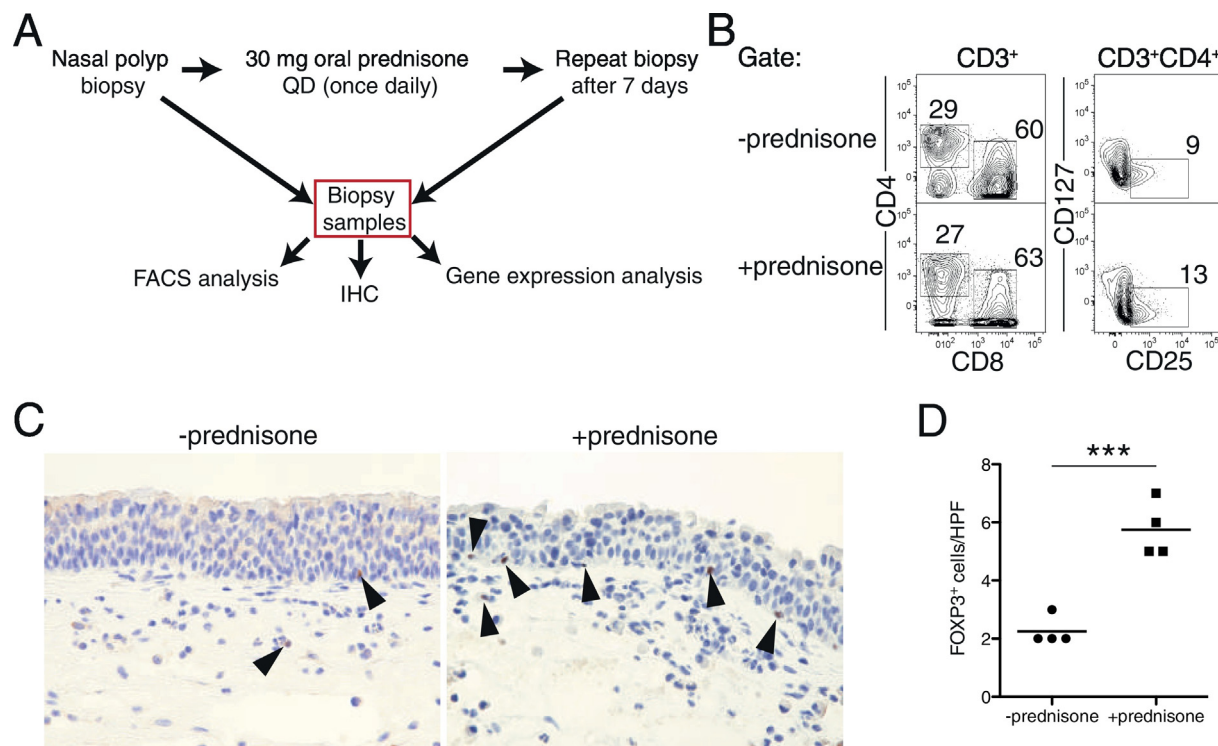
Fig. 3. Biopsy of individual CRSwNP patients pre- and post-GC treatment confirms preserved trend for T_{reg} cell expansion within NPs. A. Schematic depicting NP acquisition strategy for individual patients. NP biopsies from four patients were taken with informed consent during the patient's preoperative clinical appointment. Following a one-week course of oral prednisone (30 mg by mouth daily), a second NP tissue biopsy was obtained from the same patients in the office or operating room. NP biopsy samples were subsequently analyzed using FACS, immunohistochemistry (IHC), and gene expression analyses. B. Representative FACS plot showing an increase in T_{reg} cells ($CD4^+ CD25^{hi} CD127^{lo}$) in NPs following GC treatment within the same individual patient. This plot is representative of four independent experiments from four individual CRSwNP patients. C. Immunohistochemical staining showing an increase in $FOXP3^+$ T_{reg} cells following GC treatment in paired NP biopsies. Nuclei are shown in blue, $FOXP3^+$ cells in brown, and arrows denote $FOXP3^+$ cells. Note the increase in $FOXP3^+$ cells following GC treatment in the NP section (right). D. Quantitative analysis of $FOXP3^+$ cell numbers from immunohistochemical staining in NPs before and after GC treatment. Data are representative of four independent experiments from four different patients (pre- and post-steroid biopsies). Horizontal bars indicate mean. ***, $P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

after GC administration (Fig. 3D). These data suggest that the increase in T_{reg} cells following GC treatment is not patient specific, but rather a global trend among all CRSwNP patients.

3.6. Comparative chemokine expression profiling in NPs in the context of GCs

Given the positive effect seen in the same patient and to eliminate confounding variables, additional gene expression studies were performed on NP biopsies from the same patients before and after a 1-week course of GC therapy. To address whether chemokines are involved in the migration of T_{reg} cells into NPs following steroid treatment, we performed gene expression analyses on whole NP homogenates using a microarray containing all known human chemokines and chemokine receptors. CCL4, CCR4, CXCL1, and CXCL2 were found to be highly upregulated transcripts within NP samples upon exposure to GCs compared to NPs from the same patients used for the studies shown in Fig. 3. In contrast, CXCR1 expression was drastically reduced following steroid treatment in the polyp tissues (Fig. 4A).

3.7. Increased CCL4 expression in NPs from GC-treated CRS patients

To confirm these findings, qPCR analysis was performed with these five genes, and CCL4 was again identified as significantly increased from NPs following GC treatment (Fig. 4B). In correction with these data, CCL4 total protein titers were found to be increased in NP biopsies taken from the same patients before and after GC treatment as measured by ELISA (Fig. S5). Chemokines are soluble peptides involved in the orchestration of immune responses by interaction with chemokine receptors on their target cells. CCL4 has been previously described as

one of the most potent chemoattractants of $CD4^+ CD25^+$ T_{reg} cells in autoimmune diseases [29]. Furthermore, high concentrations of CCL4 can be detected at inflamed tissue sites [30]. The role of CCL4 in inflammatory upper airway mucosal disease such as CRS, however, remains undefined.

3.8. High CCL4 expression correlates with localized increases of T_{reg} cells in GC-treated NPs

To test whether T_{reg} cells migrated into NPs via CCL4 chemotaxis, we first analyzed the migratory capacity of T_{reg} cells from peripheral blood. Using established chemotaxis assays [29], $CD3^+ CD4^+$ T cells and T_{reg} cells migrated to an environment enriched with exogenous CCL4 (Fig. 5A, B). This result was in agreement with previous findings, in which CCL4 has been implicated in the specific migration of human $CD4^+ CD25^+$ T_{reg} cells [31]. To elucidate possible migratory behavior of T_{reg} cells into NPs, we added CFSE-labeled PBMCs to the top chamber of a 2-well migration system with NP tissues placed at the bottom. A diagram depicting PBMC migration in the presence of freshly harvested NPs from GC-treated patients can be seen in Fig. 5C. In this assay, T_{reg} cell migration was markedly augmented towards GC-treated NPs, which inherently produce elevated levels of CCL4, compared to migration seen towards NPs from GC-naïve patients.

It has been shown that T_{reg} cells preferentially express CCR4 compared to conventional T cells in normal human blood [32,33,31]. CCR4 is more commonly known as a receptor for CCL17 and CCL22, but with moderate affinity for CCL4 [31]. To determine whether the migration of T_{reg} cells can be mediated by CCR4 chemokine receptor interactions, we added a small molecule antagonist to CCR4 into the migration medium to transiently block T_{reg} chemotaxis. GC-naïve NPs did not elicit a
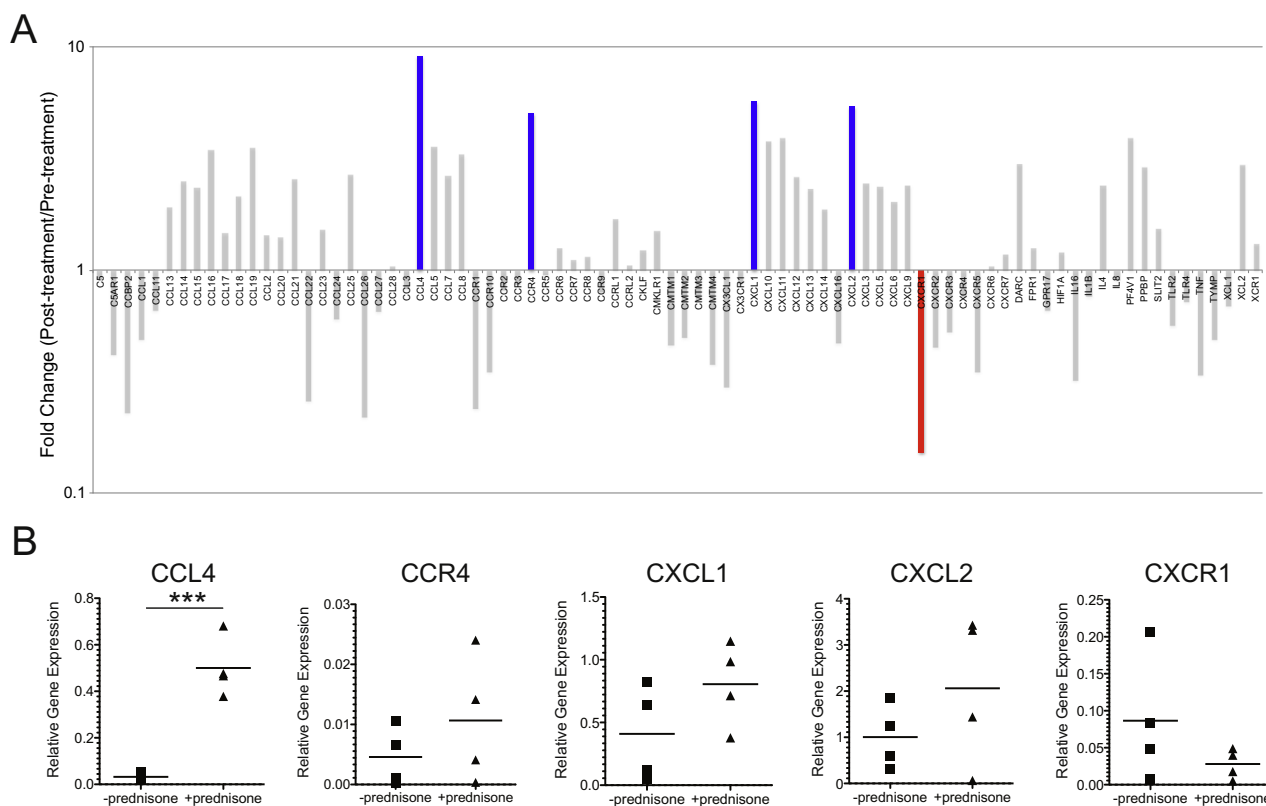
Fig. 4. Administration of GCs causes selective chemokine upregulation in NPs. A. PCR array data depicting the expression of 84 genes that encode chemokines and their receptors in NPs from the same patients before and after a 1-week course of GC treatment. Bar graph depiction shows an upregulation of several chemokines and receptors following a one-week course of oral GCs. CCL4, CCR4, CXCL1, and CXCL2 were selectively increased following steroid treatment within NP tissues (blue bars) with CXCR1 (red bar) the most down regulated gene. Data is representative of four independent experiments from four different patients (pre- and post-steroid biopsies). B. qRT-PCR analysis for mRNA transcripts of CCL4, CCR4, CXCL1, CXCL2, and CXCR1 from NP biopsies as measured by SYBR Green. Expression of the housekeeping gene GAPDH was used as an internal control to normalize results. Horizontal bars represent mean. ***, $P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

significant migratory response from T_{reg} cells, but both the number of migrated $CD3^+ CD4^+$ T cells and T_{reg} cells moderately decreased upon addition of CCR4 antagonist (Fig. 5D, F, G). With the addition of GC-treated NPs from the same patient into the migration assay, there was a statistically significant reduction in T_{reg} cell chemotaxis, suggesting that possible chemokine interactions might affect the migratory capacity of T_{reg} cells into the NP (Fig. 5E, F, G). Furthermore, the migration of both $CD3^+ CD4^+$ T cells and T_{reg} cells are significantly inhibited only when the CCR4 antagonist is added to medium containing either CCL4 or GC-treated NPs (Fig. 5F, G). Due to inherently low CCL4 expression in untreated NPs, CCR4 inhibition does not have a profound effect on the migratory capacity of both $CD3^+ CD4^+$ T cells or T_{reg} cells as expected (Fig. 5F, G). These experiments demonstrate that the migration of human T_{reg} cells from blood is enhanced when in the presence of CCL4 or GC-treated NPs, and that a CCR4 antagonist can partially impede this migratory behavior.

4. Discussion

Upper airway mucosal tissues interface with the external environment, and have developed complex immune networks between dendritic cells, mast cells, conventional T cells, B cells, and T_{reg} cells, the latter being the most understudied [34–38]. Chronic rhinosinusitis (CRS) itself is a complex disorder of this upper airway epithelium, with contributing elements from infectious and inflammatory agents at its core etiology. Here, we focused on the major subpopulation of CRS patients harboring NP burdens (CRSwNP) in order to better understand discriminating immunological characteristics present within specific NP inflammatory tissues when compared to the surrounding adjacent, but uninvolved, ethmoid sinus mucosa and the peripheral

blood (Fig. 1). This level of simultaneous analysis of resident immune cells from three human tissue subsites has been infrequently utilized, due to the natural limitations, time, and expense of accessing human tissues from the surgical theater. Without convincing murine, or other, animal model correlates of CRS studies on human tissues are essential to propel improved understanding of CRS immunity.

Therapeutic use of oral steroids served as a primary modality for uncloaking the findings presented herein, and specific functional alterations in the immune compartment have been observed in a number of other inflammatory disease states following GC treatment. Our FACS findings initially utilized the same cohort of patients as previously published by our group [14], using independent antibody staining sets and flow cytometry calibration. Once we had confirmed the trends in T_{reg} cells, however, vertical studies to better dissect this population and T_{reg} cell behavior were then conducted in additional, freshly harvested NP tissues. In this study, we found a selective increase in T_{reg} cells in nasal polyps from CRSwNP patients following GC treatment with no differences observed in those with aspirin-exacerbated respiratory disease (AERD) (Figs. 1 & S1). To better characterize these T_{reg} cells, we analyzed surface marker expression before and after GC treatment.

Activation of T lymphocytes is associated with increased expression of CD39 and GITR [39,40], although the latter has not been previously demonstrated for T_{reg} cells. In the present study, an increase in GITR expression was seen following GC treatment (Fig. 2A). Furthermore, GC therapy resulted in an increase in T_{reg} cells expressing an activated, memory ($CD45RA^- CD39^+$) phenotype (Fig. 2B). The skewed presence of an activated T_{reg} cell phenotype following steroid treatment suggests that T_{reg} cells might play a unique role in regulating inflammatory cues in nasal polyposis, as sinonasal outcomes are noticeably improved following GC therapy [41]. Alterations in T_{reg} cell surface marker
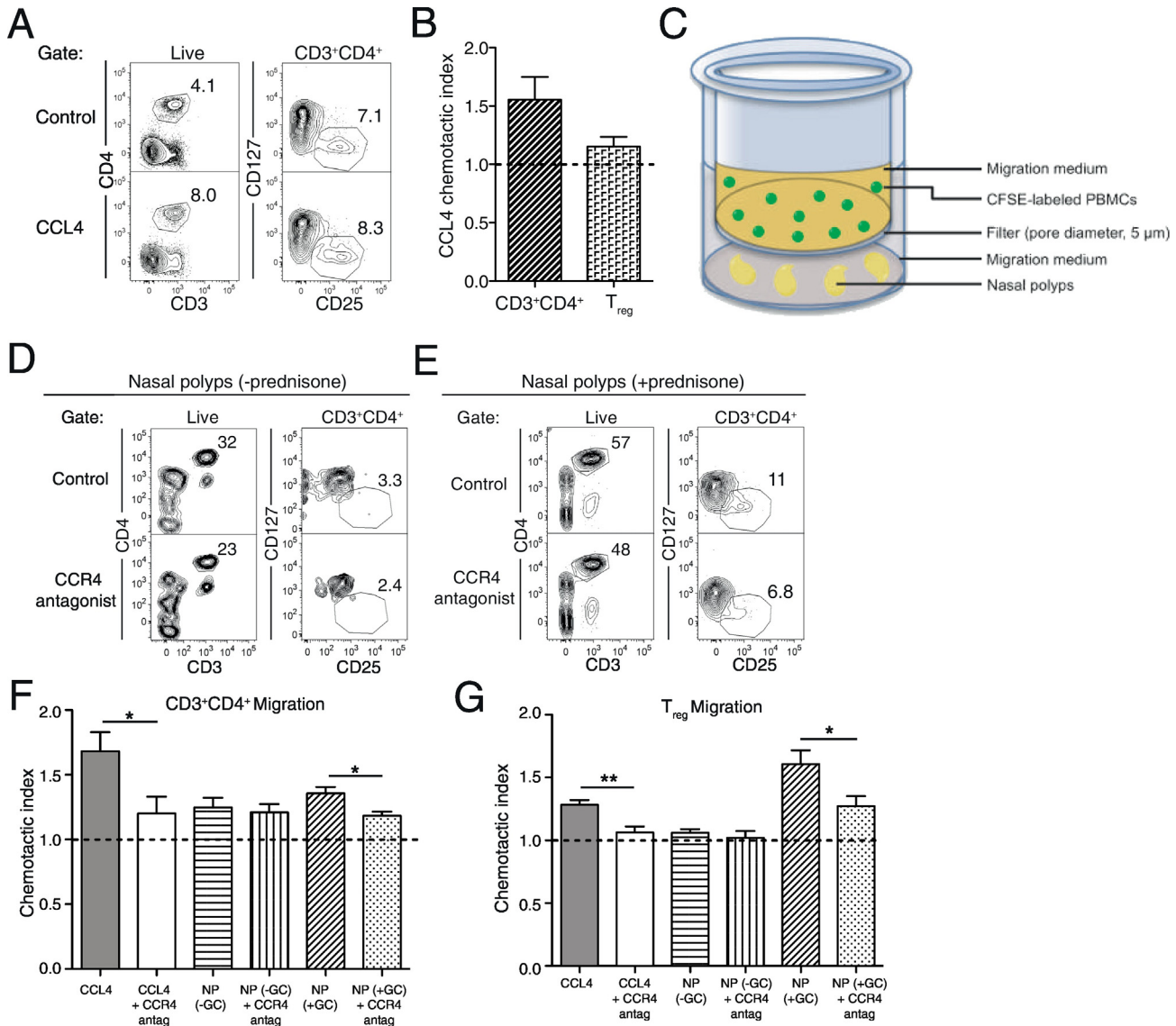
Fig. 5. Chemokine changes related to T_{reg} cell expansion in NPs. A. Representative FACS plot depicting chemotactic response of PBMCs to CCL4. After 3 h, T_{reg} cells (CD4$^+$CD25^{hi}CD127^{lo}) migrate into medium containing CCL4. Data depicted are representative of three independent experiments. B. Bar graph depicting the migratory responses of PBMCs to CCL4. Incubation with CCL4 results in an increase in migration of both CD4$^+$ cells and T_{reg} cells. Chemotactic index was defined as the number of cells migrated in response to CCL4 divided by the total number of spontaneously migrated cells. Dotted line indicates threshold for migratory capacity. The experiment was performed in triplicate, and results are expressed as the mean percentage of specific migration ± SD in response to CCL4. Data is not significant by unpaired t-test. C. Schematic depicting PBMC migration in the presence of freshly harvested NPs from GC-treated patients. D. Representative FACS plot depicting chemotactic response of CFSE-labeled PBMCs in the presence of a CCR4 antagonist and GC-naive NPs. Incubation with NPs in the presence of 10 nM CCR4 antagonist results in a slight decrease in the percentages of both CD4$^+$ cells and T_{reg} cells compared to control. E. Following a one-week course of oral prednisone, biopsied nasal polyp tissues from the same patient shown in (D) were used to determine the chemotactic response of PBMCs in the presence of a CCR4 antagonist. Incubation with steroid-treated NPs in the presence of 10 nM CCR4 antagonist results in a significant decrease in the percentages of both CD4$^+$ cells and T_{reg} cells compared to control. F. Bar graph depicting the migratory responses of peripheral blood CD3$^+$CD4$^+$ T cells in the presence of CCL4, CCR4 antagonist, or GC-treated NPs. Results are expressed as the mean percentage of specific migration ± SD in response to stimulus. n = 3 experiments; *, $P < 0.05$. G. Migratory responses of peripheral blood T_{reg} cells in the presence of CCL4, CCR4 antagonist, or GC-treated NPs. The experiment was performed in triplicate, and results are expressed as the mean percentage of specific migration ± SD in response to stimulus. * for $P < 0.05$, ** for $P < 0.01$.

expression following steroid therapy has also been seen by increases in latency-associated peptide (LAP) following four weeks of GC therapy in asthmatic patients [42]. Short-term dexamethasone administration can also amplify IL-2 dependent expansion of T_{reg} cells, enhancing their capacity to suppress experimental autoimmune encephalomyelitis (EAE) in murine models [28]. But the reliable and significant expansion of T_{reg} cells in every steroid-treated NP patient studied herein was not anticipated, especially given the reduction of granulocyte populations from NPs upon steroid exposure [14]. Finally, the T_{reg} cell recruitment induced a functional immunosuppression in the setting of NP exposed to GCs (Fig. S4), which can ultimately evoke distinct functional outcomes in the nasal upper airway mucosa. As GC therapy is a mainstay of treatment for upper airway disease, elucidating the direct effects of

steroid treatment on T_{reg} cell activity and migration is paramount to better understand the wider sphere of mucosal inflammation and immunity in human disease.

The increase in T_{reg} cells following GC treatment in CRSwNP patients works through recruitment to nasal polyp sites, as proliferation assays revealed no change in Ki67 expression in T_{reg} cells following GC treatment (Fig. 2C). We would anticipate T_{reg} cell recruitment to most readily arise from the adjacent ethmoid sinus mucosa, and indeed slight declines in T_{reg} cell numbers were seen in many ethmoid tissue specimens upon GC treatment (Fig. S1). As chemokines have been implicated in the pathogenesis of nasal polyposis [43], we next chose to explore the role of various chemokines in T_{reg} cell migration following steroid administration.

Using chemokine gene expression profiling, we discovered that selected chemokines, notably CCL4, are significantly induced within diseased NPs upon systemic steroid treatment of patients, and confirmed this at the translational level. T_{reg} cells exhibit a distinctive receptor profile for chemokines, with homing receptors CCR4 and CCR8 linked to inflammatory cell recruitment [31,16]. In the absence of GC treatment, suppressor of cytokine signaling 3 (SOC3) has been implicated in the regulation of FOXP3$^+$ T_{reg} cells in human nasal polyps [44]. In pulmonary diseases, T_{reg} cells have been shown to migrate to inflamed respiratory tissues via CCR4 to attenuate the severity of airway allergic inflammation [45]. In the present study, an increase in CCR4 expression was found on T_{reg} cells following prednisone administration by FACS (Fig. 2A). CCR4 is a known receptor for both CCL17 and CCL22, and T_{reg} cells appear to preferentially express CCR4 in both mice and humans [31,33]. It should be noted that *in vitro* studies have demonstrated that 20–40% of T_{reg} cells expressing CCR4 show specific migration in response to both CCL17 and CCL22, while 20% of T_{reg} cells show specific migration to CCL4 [31]. Though only modest affinity for T_{reg} cells to CCL4 has been demonstrated, this interaction should not be diminished. As is well described in the literature, CCL4 is a known ligand for CCR5. In our chemokine array, in which NP biopsy samples were taken from the same patient both prior to, and following, a 1-week course of GC treatment, no significant change in CCR5 expression was noted, while a selective increase in both CCL4 and CCR4 gene expression was detected in all patients. No shifts in either CCL17 or CCL22 expression were seen (Fig. 4A). Though a strong CCL4-CCR4 interaction might not be present, T_{reg} cells expressing CCR4 are increased following GC treatment and CCL4 expression elevated in NP biopsies following systemic steroid therapy.

Our findings suggest that the migration of T_{reg} cells from the peripheral sites into aberrant mucosal tissues, such as NPs following steroid exposure, occurs through induced CCL4 expression (Figs. 4B and S5), and that previously undescribed chemokine interactions in CRSwNP patients potentially exist in these upper airway tissues. A recent study by Fundová et al. has found an upregulation of both CCR1 and CCR3 in NPs, suggesting that chemokine signaling may contribute to granulocyte recruitment in CRS [46]. Chemokine-induced T_{reg} cell migration has been widely implicated in a number of both autoimmune and inflammatory diseases, including impaired CCR6/CCL20 chemokine interactions in multiple sclerosis (MS) patients [47,48], CCL28/CCR10 activity in hepatitis, and CCR4-dependent T_{reg} cell migration from draining lymph nodes into cardiac allografts in cardiac transplants [49,50]. Our data represent strong evidence for increased chemokine expression in T_{reg} cells following GC treatment in CRSwNP disease, but clearly improved understanding of chemokine/ligand interactions in the upper airway, in comparison with other tissue sites, requires increased investigation.

Finally, recruitment of T_{reg} cells from the periphery into inflammatory mucosal NPs appears to be central to the anti-inflammatory activity of GCs in CRS upper airway mucosal disease. From findings in gastrointestinal mucosa, T_{reg} cells are critical for immune homeostasis, with T_{reg} cell deficiency leading to inflammatory bowel disease [51], and mutations in the FOXP3 transcription factor resulting in severe intestinal inflammation [52]. Interestingly, in awareness of the prohibitive side effect profile of steroid use, use of antigen-specific T_{reg} cellular therapy has been proposed for targeting of disease antigens [52], with phase I clinical trials underway to assess the ability of *ex vivo*-expanded T_{reg} cells to prevent graft-versus-host-disease (GVHD) after allogeneic bone marrow transplantation [53]. In the context of human mucosal inflammatory diseases such as CRS, definition and function of induced immune effectors, such as T_{reg} cells, following GC treatment remains largely understudied. We speculate that these cells may in fact derive from the adjacent ethmoid sinus tissue bed, given slightly depressed ethmoid T_{reg} cell numbers in ethmoid tissue upon GC exposure (Fig. S1).

Inherent limitations of this work include the limited human tissue sample sizes available for study, and the inherent heterogeneity between patients and the human upper airway tissue samples retrieved in some of our experiments. While these data suggest that GC treatment upregulates CCL4 within NPs and likely leads to T_{reg} cell-specific recruitment into NP tissue sites the identity of CCL4-producing cells within NPs is unclear at this time. Several possibilities have been proposed in the literature, including B cells and APCs [29]. Further understanding of the role of, and pathways accessed by, T_{reg} cells in the nasal upper airway mucosa will improve our understanding of immunity at diseased mucosal epithelial beds. Additionally, the higher order consequences of T_{reg} cell migration within the NP bed, including shifts in cytokine and transcriptional profiles, will be of interest in future studies. Taken together, these data provide a unique window into aspects of cellular dynamics and immunoregulation for this common inflammatory upper airway mucosal disease.

Conflict of interest

Acknowledgments

Appendix A. Supplementary data

The online version of this article contains supplemental material. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.clim.2017.02.002.

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