Study title: Role of IL-5R Signaling in Non-eosinophil Upper Airway Cells in Chronic Rhinosinusitis with Nasal Polyposis

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Study Protocol and Statistical Analysis Plan on following pages.

Study Protocol and Statistical Analysis Plan

Research Plan/Approach:

Subject selection and recruitment.

These observational clinical studies will be conducted on individuals with AERD and CRSwNP who are referred to the BWH Allergy or Otolaryngology Clinics. For the surgically excised sinus tissue, patients undergoing sinus surgery will be screened to determine if they meet criteria: a diagnosis of CRSwNP, no history of cystic fibrosis or primary ciliary dyskinesia, and no unilateral nasal polyposis. Patients for this study will also be excluded if they are on any targeted respiratory biologic therapy, or on chronic oral steroids or other immunosuppressive agents. Those who meet criteria and want to participate will sign informed consent as approved by the Mass General Brigham IRB [the research proposed here is already covered under our existing IRBs, with #2019P001829 (Collection and Analysis of Healthy Sinus Mucosa and #2003P002088 (Analysis of Cells and Mediators from Aspirin-Tolerant and Aspirin-Intolerant Donors)].

Subjects will be between the ages of 18-65 years old, and we will aim to have equal numbers of male and female participants for each of the research approaches proposed below. A full physical exam, along with pertinent medical history, concomitant medications, prior history of response to medications, and prior surgical history will be collected for all participants.

For some portions of the proposal, tissue and cell samples have already been collected and are available for analysis in our tissue repository of human surgical samples (Aim 1a), and for others (Aim 1b and 2) all samples will be prospectively collected.

SPECIFIC AIM 1: Characterize the plasma cell subsets, and the functional significance of their IL-5Rα, in nasal polyps from patients with CRSwNP and/or AERD.

Overview. Nasal polyp tissue from patients with AERD contains a distinct population of plasma cells when compared to CRSwNP, marked by increased *IL5RA* expression. In this Aim we will test the **hypothesis** that ***a unique subset of IL-5-responsive nasal polyp plasma cells drives the proliferation of sinus tissue plasma cells and the production of pathogenic antibodies in the respiratory tract of patients with CRSwNP and AERD***.

To identify transcriptional, cytofluorographic, and functional characteristics of plasma cells from nasal polyps in AERD and CRSwNP, we will employ two approaches, (1) RNA sequencing and (2) functional studies. We will then use qPCR and flow cytometry to verify the results on a set of markers selected based on pathobiologic interest, including IL-5Rα.

Sub-Aim 1a. Use RNA sequencing to characterize the transcriptional profile of nasal polyp plasma cells in nasal polyps from patients with AERD and aspirin-tolerant CRSwNP.

Methods. Sinus tissue from 6 subjects with AERD and 6 subjects with aspirin-tolerant CRSwNP has been obtained. Subjects were be matched for age, gender, and intranasal corticosteroid use. Freshly excised tissue was digested into a single cell suspension. Using a

BD FACSAria Fusion Cell Sorter, plasma cells were purified and collected in TCL buffer for storage. Going forward, 1000 cells from each patient phenotype will be used for low-input RNA sequencing, and the remainder set aside for qRT-PCR analysis. RNA will be sequenced with Smart-seq2 using the Broad Institute Technology Labs facilities. Unbiased differential gene expression will be characterized using the Kallisto program to map reads to the genome, taking into account uncertainty in read mapping. To identify gene sets that may relate to biologically relevant differences in cellular function we will utilize linear mixed models. To confirm expression results from our RNA sequencing, we will validate the gene quantification of the top 5 differentially expressed genes within the plasma cell populations with qRT-PCR, using commercially available primers.

Anticipated Results. We expect that the transcriptional signature of the plasma cells from patients with AERD will show increased cell survival and activation. We expect that these cells will show upregulation of transcripts involved in activation of the cell cycle (*CCND2*), antiapoptotic genes (*BCL2*, *CFLAIR*), and those encoding surface receptors and adhesion molecules (*IL5RA*, *ICAM1*, *CD9*), along with downregulation of negative regulators of the cell cycle (*KLF2*, *KLF6*). We predict that the plasma cells in nasal polyps from patients with AERD will have higher *IL5RA* expression overall, and will express more *IGHG4* and *IGHE* transcripts, suggesting that the overproduction of nasal IgE in inflamed tissues may be driven in part by IL-5 signaling.

<u>Sub-Aim1b</u>. Determine the functional significance of IL-5Rα expression and IL-5 signaling on the plasma cells in nasal polyp tissue.

Overview. To exert effects, IL-5 induces phosphorylation and activation of several JAK and STAT proteins, and may be involved in regulation of transcripts involved in B-cell maturation, differentiation, and survival. While IL-5 supports murine plasma cell survival, IL-5-driven survival of human plasma cells has not been demonstrated. Our preliminary data has confirmed that IL-5Rα^{+} plasma cells are enriched in nasal polyps, suggesting a disease-specific mechanism that may sustain antibody production. Moreover, our studies suggest that IL-5 can upregulate expression of transcripts involved in proliferation, survival, migration and class switch in these cells. In this Sub-Aim, we will test the **hypothesis that IL-5 stimulation of nasal polyp plasma cells induces signaling (A) and regulates transcriptional activity relevant to pathogenetic function (B).**

Methods. (A) To determine the signaling capability of IL-5Rα on nasal polyp plasma cells, we will measure the phosphorylation status of their JAK1/JAK2 and STAT1/STAT5, which are rapidly activated upon IL-5 binding to IL-5Rα and can be surrogates of IL-5Rα signaling *in vivo*. We will prospectively collect surgical samples and digest nasal polyp tissue from 5 AERD patients and 5 CRSwNP patients into a single cell suspension as above, and sort plasma cells as CD38^{HI}/CD27^{HI}/CD138^{+}/CD20^{-}. These will be evaluated using intracellular flow cytometry for phosphorylated JAK1, JAK2, STAT1, and STAT5. We will first measure the phosphorylation levels on freshly isolated cells, to determine differences in their baseline *in vivo* status. We will also measure their phosphorylation levels following a 6h and 24h stimulation with or without IL-5 (1, 10 and 100 ng/mL), as described previously, to confirm their IL-5Rα signaling capacity. We will use BD Phosflow™ to optimize fixation and permeabilization for flow cytometric analysis of intracellular phosphoproteins. **(B)** To verify the transcriptional consequence of IL-5 signaling on plasma cells, we have already collected and sorted nasal polyp plasma cells from 5 patients with AERD and 5 CRSwNP and stimulated them for 6h with or without IL-5 (1, 10 and 100 ng/mL). The stimulated/unstimulated pairs of plasma cells have been collected in TCL buffer,

and banked for low-input RNA sequencing as above. Going forward, we will conduct differential expression analysis on IL-5-stimulated and unstimulated plasma cells using sample pairing, with a linear model framework. Our criterion for transcripts induced or repressed by IL-5 is that genes are more than twofold changed and obtain a false discovery rate of <1%.

Anticipated Results. (A) Plasma cell populations with higher IL-5Rα expression should exhibit increased phosphorylation of JAK1, JAK2, STAT1, and STAT5 and should demonstrate further increases upon stimulation with IL-5. **(B)** We expect to confirm that IL-5 stimulation of nasal polyp plasma cells induces expression of *CCND2, JAK1, IKZF1*, *ZBTB44*, *OTG, RAP1B*, and *RAC2*, as well as signatures reflecting activation and anti-apoptotic signals (*BCL6, AID, BLIMP-1*).

Statistical Analyses. With a sample size of 6 AERD subjects and 6 CRSwNP subjects, we will be powered at >90%, at a 0.05 level of significance, to detect a difference in antibody-secreting cells that express IL-5Rα+ between subjects with AERD and CRSwNP. The data will be analyzed as described in Aim 2.

Pitfalls and Alternative Approaches. Although the proposed work is largely hypothesis based and furthers recent preliminary observations made by our group, low-input RNA sequencing allows for unbiased analyses. Thus, while we focus on the role of IL-5Rα$^+$ plasma cells in CRSwNP and AERD pathogenesis, our approach also allows us to identify a range of differentially expressed genes and to broadly phenotype nasal polyp plasma cells.

<u>SPECIFIC AIM 2</u>: Define the IL-5-elicted molecular features of upper airway nasal epithelial tissue from subjects with healthy sinus mucosa, CRSwNP, and AERD.

Overview. Bronchial epithelial cells and human sinonasal tissue epithelial cells express a functional IL-5Rα. Further, *in vivo* inhibition of IL-5 leads to transcriptional modification of upper respiratory tract epithelial cells. In this aim, we will test the ***hypothesis that IL-5 actions on the human upper airway epithelium to reduce cell-to-cell adhesions by downregulating tight junction pathways and impacting transcripts related to cell-to-cell adhesion.***

Methods. We have already established a protocol that for the first time, shows the development of IL-5Rα on upper airway (nasal) epithelial cells. Sinonasal epithelial cells expanded to an air liquid interface culture begin to express detectable levels of IL5RA by week 2 and appear to reach maximal IL5RA transcript expression after 4 weeks in culture, which parallels their development of cilia and their expression of FOXJ1, a transcript that directly regulates genes involved in ciliary structure assembly and motility **(Figure 5)**.
Using this culture system, we will now explore the function of IL-5 signaling on these cells. Sinonasal epithelial cells will be harvested from 10 donors with CRSwNP, 10 donors with AERD, and 10 healthy donors undergoing correction of upper airway anatomic abnormalities. Epithelial cells will be expanded to passage 3 (week 4-5) in an air liquid interface culture and will be stimulated with IL-5 at 3 increasing doses for 24–48 hours. Additionally, to assess any possible chronic effects of IL-5, a portion of the air liquid interface cultures will be grown in low-dose IL-5 added into their media from week 1 (in order to more closely approximate the environment of respiratory inflammation with the constant presence of IL-5 that exists in the sinuses of patients with CRSwNP).These sets of cultured and chronically and/or acutely stimulated or unstimulated epithelial cells will then be stored for RNA and protein measurements. They will be assessed by qPCR for expression of *IL5RA, CDH1* (E-cadherin)*, CAV1* (caveolin-1)*, CAV2* (caveolin-2)*, EGFR* (epidermal growth factor receptor), *CXCLA,*

CXCL1, IL10RA, IL1RL1 (ST2), and *IL6R*, as these transcripts are known to be impacted by IL-5 stimulation in the lower airway. We will also measure IL-5Rα protein levels by Western-blot in these epithelial cells. To more broadly assess the effects of IL-5 signaling on human nasal polyp epithelial cells and to assess for yet-unidentified impacts of IL-5/IL-5Rα signaling on these cells, we will collect IL-5 stimulated/unstimulated sinus tissue epithelial cell pairs for bulk RNA-sequencing with Smart-seq2.

Anticipated results. We suspect that the sinus tissue epithelial cells from diseased patients (CRSwNP and AERD) will express higher levels IL-5Rα compared to healthy control tissue, and/or that their development of IL5RA expression will happen earlier in the maturation process compared to healthy control epithelial cells. We suspect that IL-5 stimulation of sinus tissue epithelial cells will identify downregulation of transcripts related to cellular adhesion and tight junction pathways and enrichment of a T2 immune signature.

Statistical Analyses. Differential expression analysis of RNA-seq data will be conducted using the DESeq2 package for R taking patient origin into account. Following normalization, DESeq2 will be run in a pairwise manner by comparing stimulated/unstimulated cell pairs, identifying significantly differentially expressed genes (DEGs). For pathway analyses, WebGestalt online tool will be used to identify the distribution of pre-ranked DEGs involved in specific signaling pathways in Gene Ontology Biological Processes database.

Pitfalls and Alternative Approaches. The molecular analysis of human upper airway epithelial cells is based on prior study of the human bronchial epithelial cells. However, if the pre-selected qPCR markers do not detect measurable differences between IL-5 stimulated and unstimulated cells, RNA-sequencing allows for unbiased analyses and may detect yet-undescribed changes mediated by IL-5. Further, if the molecular analyses do not identify IL-5-elicited changes in sinus tissue airway epithelium, we can plan functional phosphorylation studies on the sinus tissue airway epithelial cells as described in **Aim 1b**.

Protection of Data Security and Confidentiality:

Blinding to reduce bias. All patient-derived samples will be stored and analyzed using only a random Subject ID number, and all research will be kept blinded as to patient disease state, and all analyses of mechanistic data (flow cytometric measurements, qPCR measurements, Western Blots etc.) will be completed prior to unblinding of research investigators.

Data security. All subjects will be assigned a unique numerical identifier, and study data will be stored in a password-secured electronic database on a dedicated server. Double-keyed entry will be used to enter the data to ensure the highest levels of validity. The two generated files are compared using a verification program that checks any discrepancies among double-keyed entry. Before being added to the database, the data undergo further within-form and across-time verifications to assure completeness, accuracy, and participant identification number. Any errors in coding or keying are corrected promptly. Questionable data that need additional review are sent to a separate file and then resubmitted to the validation program after problem resolution. Nightly backups of all data files are made. All database files can be imported in SAS software for validation and data analysis.

Data confidentiality. All data will remain confidential within the investigation site. A record of study subjects entered will remain strictly confidential and will be coded only by a study identification number assigned to each subject at the time of enrollment. While the study is in progress, identifying information will be maintained to enable collection of all follow-up data.

All subject CRFs will be coded with a unique patient identifier, which will identify that subject only at the investigational site staff level. All direct subject contact (informed consent, subject screening and study procedures) and relevant confidentiality protections will be the responsibility of the PI. There should be no risk to subject confidentiality because all records associated with this study will be confidential within the study staff environment within the AERD Center. Safety precautions, password protection and encryption of data, will ensure that electronic systems will not be a risk to subject confidentiality.

Confidentiality of genetic data information. Tissue samples will be taken, from which cell populations will be isolated for genetics studies. The relevant tissue specimens and isolated cell populations for the genetics studies will be labeled with a unique numerical identification code. Subject name or other identifiers will not be placed on the genetics specimens. The key to the code will connect the subject's name to the subject's study information and specimens. The key to the code will be kept in a separate electronic database file and subject's genetic specimens will always remain coded and their identity will remain confidential.