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Title: Analysis of mucosal immune dysregulation and safety and tolerability of endoscopic topical steroid therapy for Long-COVID hyposmia: randomized, double-blinded pilot study

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Abstract

Background

Millions of people exhibit olfactory dysfunction years after acute SARS-CoV-2 infection. Evidence suggests unresolved olfactory epithelial inflammation may perturb function. Here, we report (1) data from human olfactory biopsies processed for T cell studies, and (2) outcomes from a pilot clinical trial evaluating endoscopic delivery of beclomethasone to the olfactory cleft for improving olfaction in post-COVID hyposmia.

Methods

Biopsies from post-COVID hyposmia and control subjects underwent single-cell T-cell receptor (TCR) sequencing. In a separate outpatient cohort (Duke Rhinology Clinics), we conducted a randomized, double-blind, placebo-controlled pilot trial. Eligible adults (≥18 y) had ≥3 months post-COVID smell loss confirmed by Smell Identification Test (SIT). Participants were randomized 1:1 to endoscopic delivery of saline or beclomethasone via dissolvable sponge; repeated at 2 weeks. The primary outcome was SIT improvement ≥4 points at 1 month; secondary at 3 months. Study recruitment ran Sept 15, 2023–June 18, 2024.

Results

Biopsies show no evidence of SARS-CoV-2 or EBV/HHV-6 reactivation and demonstrate clonally expanded, pro-inflammatory T-cell subsets. Fifteen subjects are randomized (beclomethasone n=7, saline n=8); 13 are analyzed (6 and 7). At 1 month, SIT improvement occurs in 66.7% (4/6) vs 28.6% (2/7) (risk difference 38.1%, 95% CI 2–97%; risk ratio 2.14, 95% CI 0.73–7.79; p=0.28). At 3 months, rates are 66.7% vs 42.9% (RD 23.8%, 95% CI –17–80%; RR 1.74, 95% CI 0.52–6.5; p=0.50). No adverse events are reported.

Conclusions

Human olfactory TCR-seq implicates local T-cell inflammation without local viral reservoirs. Directed, endoscopic topical steroid therapy is feasible and safe, with a non-significant trend toward improved olfaction, supporting larger trials. Funding: NIH DC020172, American Academy of Otolaryngology–Head and Neck Surgery.

Plain language summary:

Many people continue to lose their sense of smell long after COVID-19. We studied tissue from the smell region in the nose and found no signs of lingering virus, but we did find overactive immune cells that may cause lasting inflammation. We also tested a new way to place steroid medicine directly in the smell region using a small dissolvable sponge. The procedure was safe, and some patients improved, though the study was too small to show clear benefit. These results suggest the immune system, rather than the virus itself, may drive long-term smell loss, and that the new treatment is safe and worth testing in larger studies.

Introduction

Long COVID, defined as symptoms lasting more than 3 months after acute infection and affecting >400 million people globally[1], involves multiple organ systems with potentially debilitating neurological, cardiovascular, pulmonary, and autoimmune consequences [2,3]. Among neurologic sequelae, sensory impairments including olfactory dysfunction have driven efforts to define the underlying pathobiology. Currently, effective treatment options are lacking for those suffering from persistent hyposmia months to years after initial SARS-CoV-2 infection.

Among potential mechanisms broadly driving long COVID pathology, models support persistent reservoirs of SARS-CoV-2 [4], reactivation of latent viral pathogens such as immunotropic herpesviruses EBV and HHV-6 [5-7], molecular mimicry of viral antigens associated with elevated levels of circulating autoantibodies [8], and microvascular complications leading to poor tissue perfusion [9]. However, mechanisms specifically driving long-COVID-related chemosensory dysfunction remain unclear. It is well established that inspiration of the SARS-CoV-2 virus infects cells along the respiratory tract expressing viral entry genes, including the non-neuronal sustentacular cells of olfactory epithelium (OE) [10,11]. Lining the superior portion of the nasal cavity, the OE balances sensory and barrier epithelial functions, continually replacing damaged cells including neurons via signaling interactions involving basal epithelial stem cells and local immune cell populations [12,13]. However, severe damage or persistent pro-inflammatory signals can perturb OE homeostasis and function [14]. A provisional model in which unresolved post-COVID local inflammation drives long COVID smell loss warrants further investigation[15]. Other non-mutually exclusive models include persistent gene expression changes in olfactory neurons, or processes impacting the olfactory bulbs or brain directly. TCRseq analysis of human olfactory tissue shows no evidence of a local SARS-CoV-2 reservoir or reactivation of latent viruses. Instead, clonally expanded T cell subsets display a pro-inflammatory profile, including recognition of a human antiviral defense protein. A pilot clinical trial of endoscopic delivery of a long-acting steroid to the olfactory cleft is safe and feasible, with trends toward improved olfaction at 1 and 3 months, supporting larger phase trials.

Methods

Human biopsies

All human studies were performed under protocols approved by the Institutional Review Board of Duke University. Biopsy samples reported here were collected under IRB protocols 00088414 and 00105837.

Biopsy subjects were recruited from Duke Department of Head and Neck Surgery & Communication Sciences Rhinology clinics. Olfactory function was measured using the University of Pennsylvania Smell Identification Test (SIT; Sensonics, Inc, Haddon Heights, NJ, USA). The SIT is a validated 40-item test of olfactory identification[16]. Patient demographics, including

duration of hyposmia/anosmia since acute COVID-19 infection, as well as relevant treatment history were recorded (Table 1, Table S1). Control samples included olfactory tissue biopsies obtained from normosmic individuals.

Subjects were administered the SIT prior to tissue collection, to assess olfactory function. Olfactory mucosa biopsies were either surgically excised in the operating room from patients undergoing transsphenoidal surgery for resection of a benign pituitary tumor, or were collected in the clinic using cytology brush biopsy. Briefly, for surgical biopsies, using endoscopic visualization olfactory cleft mucosa was sharply incised using a surgical 12-blade and elevated from underlying bone gently with a freer elevator, and then excised with a through-cutting ethmoid forceps. For nasal cytology brush biopsies, tissue was collected in the outpatient clinic. Awake patients were treated with topical nasal oxymetazoline/tetracaine spray. After 3-5 minutes, biopsy was performed by gently positioning a cytology brush (Cat#4290, Hobbs Medical Inc, Stafford Springs, CT, USA) in the olfactory cleft under endoscopic visualization. The brush was rotated briefly to collect surface mucosal cells. In all cases, samples were placed into collection solution [Hibernate E medium, with 10% fetal bovine serum (FBS, all from Thermo Fisher, Waltham, MA)] on ice and processed immediately for analysis.

Cell dissociation

Surgical biopsy tissues were divided into smaller pieces sharply. All biopsies were digested as previously described [15] for 15 minutes at 37°C with an initial 2.5 ml enzyme cocktail comprised of Dispase, Collagenase (1 mg/ml), EDTA (1mM), Papain (2 mg/mL) activated with beta-mercaptoethanol, DNAse I (all from StemCell Tech, Vancouver, BC, Canada) and N-acetyl cysteine 5 µg/ml (Sigma, St. Louis, MO, USA) with frequent gentle trituration. After 15 minutes, an equal volume of 1x Accutase (StemCell Tech) was added, and samples were incubated for an additional 5 minutes at 37°C. At the end of 5 minutes, FBS was added. If samples still contained large pieces of tissue, they were filtered through a 250µm filter. All samples were then filtered through a 70µm filter and centrifuged 5 minutes at 400 × g. If abundant red blood cells were observed in the pellet, tissues were re-suspended in 0.5 mL of ACK (ammonium-chloride-potassium) lysis buffer (Thermo) and incubated at room temperature for 3 minutes per manufacturer's instructions. Samples were washed in HBSS, spun and resuspended in HBSS or Hibernate-E containing non-acetylated bovine serum albumin 1 mg/ml (Thermo), anti-clumping reagent 0.5 µl/ml (Gibco), and N-acetyl cysteine 5 µg/ml (Sigma) to a final concentration of 1 million cells/mL. Brush biopsies were processed similarly, but required slightly less time in dissociation enzymes and did not require an erythrocyte lysis step.

5' gene expression and TCR VDJ sequencing

Samples were processed for single cell analysis as described previously[17]. Briefly, cells were quantified with a viability stain on an automated counter (Cellaca MX, Nexcelom) and loaded onto a Chromium iX controller (10X Genomics, Pleasanton, CA) for cell capture and bar coding targeting 10,000 cells, per the 5' v2 gene expression protocol per manufacturer's instructions. Reverse transcription, amplification, library preparation, and were performed per protocol. Sequencing was performed at a depth of 300 million paired reads (NovaSeq 6000, Illumina).

Assessment of Viral Reactivation

To simultaneously measure human and viral transcripts, a custom reference genome was built by appending EBV B95-8 (GenBank accession number NC_007605.1), HHV-6A U1102

(NC_001664.4), HHV-6B Z29 (NC_000898.1), SARS-CoV2-omicron (OR939741.1), and SARS-CoV2-delta (OR939692.1) genome sequences as chromosomes to the human genome GRCh38. Positive controls for EBV-HHV6a co-infection, EBV-HHV6b co-infection, HHV6, and SARS-CoV2 were obtained from sequence read archive numbers SRX20032491, SRX20032490, SRR2071067, and SRR11181959, respectively. Reads were demultiplexed and aligned using Cell Ranger (10X Genomics). For each virus, we extracted genes from the entire viral genome from the raw count matrix, calculated mean expression for every viral gene across all cells for each patient, and stored the results in a "subject x virus" table. Finally, we log-transformed these mean values ensuring sensitivity to trace viral transcripts for visualization. The reference genome will be available for download at (https://github.com/Goldstein-Lab).

Single cell sequencing and quality control

Single-cell 5′ GEX (scRNA-seq) and αβ TCR V(D)J sequencing data from three COVID hyposmic and three normosmic control samples were demultiplexed and converted into FASTQ files using the 10X Genomics Cell Ranger v8.0.0 pipeline. Reads were aligned to the GRCh38-2024-A (Ensembl 98) human genome. Gene expression counts, barcodes, and UMIs were generated using cellranger count, while TCR sequences were assembled and annotated using cellranger vdj.

Quality control and downstream analyses were performed in Python (v3.9.13) using Scanpy (v1.9.1) following best practices for single cell analysis. Filtered feature matrices were processed as AnnData objects with sc.read_10x_mtx(), filtering was performed by excluding cells with >50,000 or <500 total counts, >8,000 gene counts, or >30% mitochondrial content. Normalization was performed with sc.pp.normalize_total() for a target sum of 10,000. For integrated analysis, multiple scRNA-seq datasets were merged using sc.concat(join="outer") before clustering. The new 6 human sequenced biopsies were integrated with human biopsies from 16 patients whose datasets have been previously published and available using GEO accession number GSE201620[15], GSE139522[18], GEO GSE184117[17].

Clustering was performed using scvi-tools (v0.17.4), which has been benchmarked for minimizing batch effects while preserving biological variability, particularly in large integrated datasets. 5000 highly variable genes (HVGs) were identified with sc.pp.highly_variable_genes() using the "seurat_v3" flavor and batch key set to the specific scRNA-seq subject sample to control for subject-level batch effects. Poisson gene selection was then performed with scvi.data.poisson_gene_selection(), using the same number of genes and batch key as in the previous step. To set up the probabilistic deep learning model, scvi.model.SCVI.setup_anndata() was run on an AnnData object containing only the top genes from Poisson gene selection. Categorical covariate keys included olfactory condition as well as biopsy sample, while continuous covariates included percent mitochondrial counts, with the batch key remaining the same. The model was trained with default parameters, an early stopping patience of 20, and 500 max epochs using model.train(). The learned latent representation was extracted with model.get_latent_representation() and stored in .obsm of the full AnnData object. Using the latent representation, neighbors were computed with sc.pp.neighbors(), followed by UMAP generation with sc.tl.umap(min_dist=0.5). Leiden clustering was performed with sc.tl.leiden(resolution=2.0). Throughout the scVI pipeline, raw count layers were used for all steps.

Additional rounds of quality control were conducted based on assessment of genes by counts, total counts and percentage of mitochondrial counts for each cluster to identify low-quality cells

or doublet cells. Prior to eliminating a low-quality cluster, we examined gene expression using known marker for olfactory epithelium cells [18], and ran sc.tl.rank_genes_groups() on the normalized data to identify marker genes for each cluster to retain biologically relevant cells. Once low-quality clusters were appropriately removed, we re-ran the scVI pipeline on the updated AnnData object.

T cell clustering and annotation

For T cell clustering analysis, the filtered feature matrix files from each of the six human olfactory epithelial samples (three COVID-19 hyposmic and three normosmic controls) processed for 5' gene expression (GEX) and TCR αβ V(D)J sequencing were loaded into Python (v3.9.13) using sc.read_10x_mtx and ir.io.read_10x_vdj, respectively. A multimodal MuData object was then created, integrating both gene expression and TCR sequencing data, with the gex and airr keys assigned to recall the 5' GEX and TCR sequencing AnnData objects, respectively. T cells were identified by selecting the intersection of barcodes present in both gex and airr. T cell clustering was performed using scVI-tools (v0.17.4) on mdata.mod["gex"], following the same quality control and scVI pipeline described above (excluding cells with >50,000 or <500 total counts, >8,000 gene counts, or >30% mitochondrial content), except using 8,000 HVGs to account for the increased variability and activation states of T cells. Dot plots were created using sc.pl.dotplot() to manually classify T cell subsets based on canonical gene expression profiles of each scVI cluster, where the dot color indicates the level of gene expression, and the dot size represents the percentage of cell expression per sample (Supplemental Fig 2a). Proportion of cell counts per cluster were first determined by grouping cells according to both cluster identity and experimental condition (Normosmic vs. Hyposmic). A contingency table was constructed for each cluster comparing the number of cells in that cluster under each condition to the number of cells in all other clusters under each condition.

TCR quality control and annotation

Python (v3.11.11) was used to load a multimodal MuData (v0.3.1) object containing both gene expression and TCR sequence data. Scirpy (v0.20.1) was used to parse and annotate TCR chain information. As part of the quality control process, multichain or orphan VJ/VDJ chains were removed, retaining only cells with valid single-chain or dual-chain receptors. Clonotypes were defined by first computing sequence identity with default parameters in Scirpy ("ir_dist") to generate nucleotide sequence distance matrices for both VJ and VDJ receptors. These matrices were used to assign clonotype identifiers ir.tl.define_clonotypes, with receptor_arms='all' and dual_ir='primary_only', ensuring that both receptor arms were considered while focusing exclusively on primary immune receptor chains. Clonal expansion metrics were calculated ir.tl.clonal_expansion. Each cell was then labeled expanded if its clonal expansion value exceeded two. Distinct expanded and total clones per T cell subset were then counted, then T cell subsets were ordered by their mean number of expanded clones, calculated across all patients and conditions (Fig. 1g).

To analyze clonotype similarity, we computed pairwise TCR distances using ir.pp.ir_dist, setting metric="tcrdist" to measure TCR sequence similarity, sequence="aa" to analyze amino acid sequences, and cutoff=15 to define the maximum allowed distance between sequences for clustering. This step generated distance matrices for both VJ and VDJ receptor chains. Next, clonotype clusters were defined using ir.tl.define_clonotype_clusters, maintaining the TCRdist

metric and considering all receptor arms receptor_arms="all" and any immune receptor chains dual_ir="any". To visualize clonotype connectivity, we constructed a clonotype network with ir.tl.clonotype_network, requiring a minimum of three cells per clonotype. This network representation grouped related clonotypes into clusters, with each fully connected subnetwork representing a distinct clonotype cluster (Supplemental Fig. 2c).

To analyze CDR3 sequence motifs across both olfactory conditions, we employed Scirpy's pl.logoplot_cdr3_motif function to generate sequence logos. For each olfactory condition we focused separately on the VJ and VDJ receptor chains. For each chain, we filtered cells to include only those with CDR3 amino acid sequences (junction_aa) of length 12. Next, we generated sequence logos with the "chemistry" color scheme, which assigns colors to amino acids based on their chemical properties. Specifically, polar amino acids (G, S, T, Y, C) are colored green; neutral amino acids (Q, N) are purple; basic amino acids (K, R, H) are blue; acidic amino acids (D, E) are red; and hydrophobic amino acids (A, V, L, I, P, W, F, M) are black. and set the to_type parameter to "information" to represent the amino acid content at each position. This methodology allowed us to visualize and compare CDR3 sequences across both olfactory conditions, providing insights into the TCR repertoire's structural and antigen binding characteristics (Supplemental Fig. 2d).

TCR antigen specificity

To infer antigenic targets of identified clonotypes, we incorporated VDJdb, a curated repository of TCR sequences annotated with experimentally verified antigen specificities. We utilized Scirpy's ir.tl.ir_query function to compare our dataset against the VDJdb reference database, employing amino acid sequence identity as the matching criterion, considering both VJ and VDJ receptor arms. We computed TCR amino acid sequence similarity by setting metric="identity" and sequence="aa" to perform exact amino acid matches against the database. For cells mapping to multiple VDJdb entries, the strategy="most-frequent" option resolved the best match by selecting the most common reference hit to a given antigen in “antigen.species” (Fig. 1d).

Gene Set Enrichment Analysis

To contrast genes differentially expressed across all OE T cells from COVID-hyposmic and normosmic individuals, we used the Wilcoxon rank-sum test in Scanpy (v1.10.4) on normalized counts, specifying normosmic as the baseline. Only highly variable genes were retained. Gene set enrichment analysis (GSEA) was performed with the decoupler package (v1.9.2) on the 2024 c2 reactome gene set (https://www.gsea-msigdb.org/gsea/msigdb/human/genesets.jsp?collection=CP:REACTOME). We filtered all reactome pathways to include only those that contained 15–500 genes for a given pathway to balance breadth and specificity. Each gene pathway set received a normalized enrichment score (NES) based on how strongly its constituent genes aligned to the ranked list. A nominal p-value was then assigned to each pathway, reflecting the likelihood of observing such an enrichment by chance. Significant pathways ($p < 0.05$) were carried forward for visualization and biological interpretation. Relevant pro- and anti- inflammatory pathways were manually bolded in Fig 1h for visualization.

Pilot clinical trial

Pilot clinical trial was approved by the Duke IRB under Pro00113299. Subjects were randomized to receive saline (placebo control) or beclomethasone treatment in double-blinded fashion. We

hypothesized that local application of anti-inflammatory beclomethasone to the olfactory cleft would inhibit local immune cell activity, leading to improved olfactory function. For pilot sample size calculation, we sought to enroll 15 subjects to evaluate feasibility of the approach. The study period lasted from September 15, 2023 to June 18, 2024. Inclusion criteria included patients male or female, aged 18 years or older who endorsed post-COVID smell loss for greater than 3 months, as confirmed by the SIT. Exclusion criteria included pregnancy or lactation, febrile illness within 1 week, treatment with another investigational drug within 3 months of enrollment, active sinonasal disease by nasal exam (including rhinosinusitis, nasal polyps), adults unable to consent, prisoners, employees or subordinates, allergy to the drug, posisep, or shellfish, as well as a history of glaucoma. Patients were randomized to the drug or placebo arm by the Investigational Drug Services (IDS) in the Duke Department of Pharmacy in a double-blinded fashion to the patient and the provider using a random number generator. The IDS then provided the drug in a syringe with an ambiguous label that was pre-filled with steroid or placebo drug in a double-blinded fashion. Researchers were unblinded only after closing the study. It is unlikely the patients could guess their treatment allocation, as the medication was dispensed directly into the sponge after placement in the olfactory cleft. Although not designed to test efficacy, we considered a 4-point improvement on SIT scores (the accepted minimal clinically important difference (MCID)) as an outcome measure. This study was conducted as a per-protocol analysis. 15 subjects were randomized, 2 were removed because one scored >30 on the pre-treatment SIT (borderline normosmic) and another tested positive for repeat COVID infection following enrollment; 13 subjects completed the study and were available for analysis. Considering sex as a variable, male and female enrollment were balanced among treatment and control groups (Table 1). Clinical trial registration: ClinicalTrials.gov: NCT05970731.

Treatment procedure: the nose was initially decongested and anesthetized with oxymetazoline/tetracaine spray. Using a 4 mm 0 degree or 30 degree rigid nasal endoscope (Karl Storz, Tuttlingen, Germany), a cotton ball soaked in oxymetazoline/tetracaine was placed at the middle turbinate tip briefly then removed. Approximately two to three 0.5-0.75 cm pieces of PosisepX sponge (Hemostasis, St. Paul, MN, USA) were then gently placed into the olfactory cleft bilaterally to maximize exposure to beclomethasone. Placement began with a dry sponge positioned at the entrance of the cleft, followed by instillation of the medication (or placebo) directly onto the sponge using a syringe. This was repeated with one to two additional sponges, which gently displaced the prior sponge posteriorly and superiorly into the olfactory cleft proper (Figure 2b shows this initial placement). This stepwise delivery ensured drug coverage along the superior cleft while avoiding aggressive packing. Importantly, we intentionally did not pack the cleft to its full capacity. The hydrated nature of the sponge allows it to conform to native tissue without exerting significant lateral force on the middle turbinate, minimizing the risk of turbinate lateralization. Of note, no participants reported pain during placement or follow-up, no epistaxis was observed, and there were no cases of iatrogenic anosmia or endoscopically visible scarring. The sponges are a biocompatible, dissolvable chitosan-based polymer. Each sponge wasinfiltrated with a total of 0.3ml of study medication per side (either 84 mcg/0.3ml beclomethasone drug or 0.9% sodium chloride placebo, obtained in randomized double-blind fashion from IDS pharmacy), via syringe with 25G 1.5 inch needle. Treatment was repeated 2 weeks later for a second dose.

Primary endpoint was a measurement of olfactory function, tested via the SIT, which was administered pre-treatment to confirm baseline olfactory dysfunction, and 1 month and 4 months

after the second treatment. At the time of designing this pilot study we were unsure if a benefit would be rapid and/or transient and therefore added a second timepoint to assess this. A secondary endpoint was the Questionnaire on Olfactory Dysfunction (QOD), a validated patient reported outcome measure, administered at the time of each SIT. Safety was assessed by patient self-report of adverse events and nasal endoscopic exams. Nasal endoscopic exams were performed at each treatment visit to monitor the overall health of the olfactory mucosa, as well as inflammation, scarring and retained sponge material. Changes in parosmia as a result of treatment were not formally assessed. The trial was not designed or powered to evaluate therapeutic response in this subgroup, and no conclusions regarding efficacy in parosmic patients can be drawn.

Statistics and Reproducibility

All sequencing data set analyses were performed in Python as described above. Plots were generated in Python using Scanpy or in GraphPad Prism 10. The analysis plan, including the use of the SIT as the primary outcome and the choice of statistical tests, was pre-specified prior to database lock. A departmental statistician was consulted to confirm the appropriateness of Fisher's exact test given the small sample size, and all analyses were conducted independently to minimize bias. Fisher's exact test (two-sided) was used to evaluate whether the proportion of T cells in each cluster differed significantly between Normosmic and Hyposmic groups. Multiple hypothesis testing correction was applied to the p-values using the Benjamini–Hochberg (FDR) method. Statistical analysis in the clinical trial study was performed using Fisher's exact test to assess drug versus placebo groups. We sought to test for an association between drug and olfactory improvement, defining ΔSIT score $\geq$4 points as MCID. Sample sizes were three COVID-hyposmic and three normosmic subjects for scRNA-seq/TCR-seq analyses, and 13 subjects (after exclusions) for the pilot clinical trial. Each biopsy represented one biological replicate, and no technical replicates were performed.

Results

Single cell TCR-sequencing from olfactory cleft biopsies

In other organ systems impacted by long COVID, T cell alterations are increasingly implicated in pathogenesis [19]. Here, we leveraged the ability to access the olfactory organ in the nasal cavity for minimally invasive biopsies to further define T cell phenotypes active in the OE of long COVID hyposmics. By integrating new samples processed for 5′ T cell receptor (TCR) sequencing to characterize the T cell repertoire (n=6 subjects) with our existing scRNA-seq datasets [15,17,18], a comprehensive olfactory cell atlas was generated (Supplemental Fig. 1, 158,014 cells, n= 22 subjects). T cells co-expressing CD3D, CD3E, and CD3G with TCR-seq data available (n=2,408 cells) were re-clustered and analyzed based on patient contribution, canonical T cell phenotypes, and olfactory status (Fig 1a-c, Supplemental Fig 2). To test whether T cells in the OE may be specific for SARS-CoV-2 or other latent viruses, we queried their TCR sequences against VDJdb, a repository containing over 30,000 paired-chain TCR sequences annotated with verified antigen targets [20]. No TCRs matched known epitopes for SARS-CoV-2, EBV, or HHV6 antigens (Fig. 1d). Of interest, in a COVID-hyposmic sample we identified a T cell clone recognizing the *Homo sapiens* BST2 antiviral defense protein (Bone Marrow Stromal Antigen 2, also known as Tetherin), consistent with potential autoimmune mechanisms. We also detected T cells across both conditions with TCRs specific to Influenza A and CMV, consistent with common

viral exposures in nasal mucosa. Next, we mapped available scRNA-seq transcripts from patients or positive control cell samples to viral reference genomes (n=15 subjects, Supplemental Fig. 3, Fig 1e). Our samples contained no detectable SARS-CoV-2, HHV4 or HHV6 transcripts, suggesting that local viral reservoirs or reactivation are unlikely.

Mapping T cell subsets from the OE of COVID hyposmic subjects revealed alterations in their T cell subset composition relative to normosmic controls. Specifically, there was a significantly increased proportion of IL-17-producing CD8+ T cells (Tc17) and natural killer T cells (NK T), accompanied by a greater diversity and expansion of unique clones within these subsets (Fig. 1f,h, Supplemental Fig. 2). To compare functional differences across all T cells between hyposmic and normosmic individuals, we performed differential expression analysis and mapped the results to canonical biological pathways. T cells from COVID-hyposmics displayed a mixed inflammatory profile, characterized by significant upregulation of pro-inflammatory (IL-1, TLR1/2, and NF-κB signaling) as well as a T helper-2 (Th2) pathway (IL-4, IL-13) relative to normosmic subjects (Fig. 1g). Together, these analyses suggest a complex, ongoing inflammatory T cell response in the OE of long COVID hyposmics, in the absence of active local virus activity.

Pilot clinical trial of a directed topical intranasal anti-inflammatory therapy for long-COVID hyposmia

Based on these findings, and prior evidence from rodent and human that local inflammation can drive olfactory impairment via multiple pathways [13-15,21], we next tested feasibility of a directed anti-inflammatory therapy in a separate cohort of long-COVID hyposmic patients. Importantly, typical patient-administered nasal sprays are inefficient at depositing medication to the olfactory region of the nasal cavity[22], likely limiting their therapeutic efficacy. Here, we performed a pilot randomized, double-blind trial in an outpatient setting to precisely place a dissolvable drug delivery sponge into the olfactory cleft via endoscopic guidance and applied the steroid beclomethasone or saline placebo (Fig 2a,b). Treatment was repeated at 2 weeks. Subjects were administered a psychophysical olfactory test (SIT) and a patient-reported outcome measure (Questionnaire of Olfactory Disorders (QOD)) at pre-treatment and one and four months post-treatment to identify the timing and duration of potential changes in smell function. Informed consent was obtained from 15 adult subjects, with 13 available for analysis (Table 1). The mean duration post-COVID19 onset was 7.3 months (range 6 to 10 months). Average pre-treatment SIT scores were similar between the steroid and saline control groups (21.71 vs 19.42, p = 0.54, Student's t-test). The SIT score, with a minimal clinically important difference (MCID) defined as ≥4, was used as the primary outcome measure [23]. Endoscopic sponge placement and medication treatment was well tolerated, with no adverse events reported, such as pain, hemorrhage, local anatomic injuries, or medication reactions. On subsequent endoscopic examination, no scarring or synechiae were noted in the olfactory cleft. While the pilot trial is not designed to test efficacy, we noted a trend towards improvement in the treatment arm (Figure 2c, Supplemental Fig 4, Supp Table 1). The proportion of subjects achieving MCID on the SIT score in the treatment group was 66.67% vs. 28.57% in control (Absolute % improved = 38.1% (95% CI: 2-97%), Relative improvement = 2.14 (0.73-7.29) at one month, p = 0.28, Fisher's exact test) The rate of improvement in the treatment group was 66.67% vs. 42.86% in control (Absolute % improved = 23.81% (-17-80%), Relative improvement = 1.74 (0.52-6.5, p = 0.50) at three months (Table 2).

Although the study showed a trend toward improvement in SIT scores, the small sample size and lack of statistical significance indicate that these findings do not support definitive conclusions about efficacy. As a proof-of concept pilot trial, we conclude that the office treatment is simple, feasible and well tolerated. Further refinements of this approach may include optimizing the dosage and timing regimen, as well as the specific anti-inflammatory compound.

Discussion

We sought here to better define T cell changes driving long-COVID hyposmia to guide a new, directed anti-inflammatory therapeutic approach. A growing body of literature has begun to define the pathobiology of long COVID, a multisystem disorder estimated to affect >400 million people globally [1], yet therapies for long-COVID olfactory dysfunction are lacking. Olfactory dysfunction is linked to malnutrition, depression, anxiety, social dysfunction and, among the elderly, increased mortality [24]. Treatment for olfactory dysfunction represents a major unmet need. An estimated 5-10% of patients may remain hyposmic months to years following olfactory dysfunction during acute COVID19 [25]; equating to millions of people. Our single-cell analysis first uncovered a dominant pro-inflammatory T-cell program in the OE, implicating local T-cell–driven inflammation as the mechanism of disease. This finding provided the rationale for the steroid arm, which was specifically designed to pharmacologically extinguish that program.

Given the pleiotropic nature of long COVID, it is unlikely that a single mechanism accounts for all symptoms. Accordingly, our findings from olfactory biopsy analysis suggesting an absence of an olfactory SARS-CoV-2 reservoir or local latent viral reactivation does not exclude the possibility that these mechanisms occur elsewhere. However, TCR-seq supports possible autoimmune mechanisms, identifying a receptor recognizing a human antigen (Figure 1d). Our combined analysis involves scRNA-seq of >158,000 olfactory cells from 9 COVID hyposmics and 13 controls (Supplemental Fig. 1), providing a robust data resource. We provide a working model in which local immune dysregulation is a common feature among long COVID hyposmic biopsies, broadly consistent with current hypotheses for long COVID pathogenesis in other systems. Future efforts to further define clonal T cell expansion in the olfactory cleft, to leverage computational modeling and database resources to identify TCR antigens, and to refine our understanding of specific OE-immune cell signaling interactions regulating epithelial repair from basal stem cells, barrier epithelial maintenance, and neuronal function will build upon these initial findings.

As a sensory epithelium accessible to endoscopic visualization, locally applied topical therapies are an attractive option for management of olfactory disorders. Several treatment strategies have been investigated with mixed results: intranasal theophylline[26] stellate ganglion block[27] each failed to improve outcomes in separate studies. A prospective cohort suggested that platelet-rich plasma injections may provide long-term improvement for a subset of patients, though mechanisms of action remain unclear[28]. Systemic steroids have been used for COVID-related smell loss but have shown inconsistent benefit in clinical studies, likely due to subtherapeutic concentrations at the olfactory cleft, while also carrying a higher risk of systemic side effects[29]. Our pilot trial using a dissolvable drug delivery sponge and the anti-inflammatory drug beclomethasone provides evidence for safety and feasibility of such an approach. The clinical outcomes here were negative, with only a non-significant trend toward improvement in olfactory test scores at one- and four-months post-treatment. These findings should be interpreted with caution, and do not support conclusions regarding efficacy, but may inform the design of

future, adequately powered trials. Importantly, this endoscopic approach was easily applied in the outpatient setting in awake subjects, requiring only brief topical anesthetic and decongestant. Limitations of this study include the pilot nature, with overall small sample sizes; and participants of our pilot trial were mostly female. However, this is consistent with findings that females are disproportionally susceptible to long-COVID smell loss [30,31]. To our knowledge, human TCR-seq data from the olfactory epithelium have not yet been reported, and we hope this initial dataset encourages further replication and investigation. Interpretation of these data may be limited by age differences between control and long COVID-hyposmics, and efforts to further expand publicly available olfactory cleft TCR-seq datasets will enable more powerful analyses. As our understanding of pathobiology evolves and newer drug agents become available, the olfactory cleft may be amenable to more potent selective targeted therapies, with local application avoiding potential systemic off-target effects.

Methods: see online Methods for full details. All necessary patient/participant consent has been obtained and appropriate institutional policies followed.

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Author Contributions:
BG designed and led the project, including obtaining funding and IRB approval. DJ, RA and BG obtained clinical samples. BG, SK conducted the pilot trial including recruitment, consent, smell testing, as well as treatment delivery. VD, SK, JF, TK, BG designed and conducted experiments and interpreted data. JF, TK, MW helped troubleshoot TCRseq experiments. VD, SK, BG generated and edited the manuscript and figures.

Data availability: All scRNA-seq and T cell receptor sequencing files are publicly available in the Gene Expression Omnibus (GEO) under accession numbers GSE290884 and GSE290883, and raw/processed data are stored on secure Duke University servers. The numerical source data for Figs. 1e–h and 2c are provided in Supplementary Data 1. All other relevant data are available from the corresponding author upon reasonable request, with responses typically provided within two weeks.

Code availability: Code developed for data analysis is available via Github at:
https://github.com/Goldstein-Lab/Kim-D-Anniballe_COVID_TCR[32]

Conflicts: BJG is co-founder of Rhino Therapeutics, unrelated to this research. Other authors have no relevant disclosures.

Figure legends

Figure 1. T cell composition, clonal expansion, antigen specificity, and pathway enrichment differences between long-Covid hyposmic subjects and normosmic controls. (**a**) T cell subset identified by scRNA-seq from biopsies, visualized by UMAP colored by subjects (Normosmic 1-3, COVID-hyposmic 1-3), (**b**) T cell cluster identities and (**c**) cell composition colored by olfactory condition. (**d**) UMAP projection of T cells, with color indicating the TCR's matching antigen species from the VDJdb. Legend shows the antigen species and the number of matching TCR sequences. (**e**) Expression of HHV4, HHV6, and SARS-CoV2 viral transcripts in samples or positive controls. Zero viral transcripts were identified in normosmics or COVID-hyposmics. (**f**) Visualization of T cell composition by olfactory condition; * $p < 0.001$, Fisher's exact test with FDR correction. (**g**) Number of unique expanded (>2) clones by T cell subset and olfactory condition. Error bars show standard error of the mean for each condition. Subsets identified as significantly upregulated in hyposmics in (f) are also bolded in (g) to denote their clonally expanded status. (**h**) Top significantly upregulated reactome transcriptional pathways across all T cells from COVID-hyposmic biopsies relative to normosmic controls. Immunological pathways are highlighted in bold; normalized enrichment scores (NES) indicate a heightened, mixed inflammatory signature. CD8 T cyt = Cytotoxic CD8 T cells, Tc17 = IL-17-producing CD8+ T cells, CD8 T rm = Tissue resident CD8 T cells, CD4 T em = Effector memory CD4 T cells, NKT = Natural killer T cells, Treg-like = Regulatory T-like cells, MAIT = Mucosal associated invariant T cells.

Figure 2. Pilot clinical trial using endoscopic directed topical therapy to the olfactory cleft. (**a**) Schematic of internal nasal cavity; olfactory cleft (highlighted blue) along anterior skull base was visualized with a rigid nasal endoscope for (**b**) placement of drug delivery sponge (asterisk) and application of drug or placebo; MT = middle turbinate, S = septum, IT = inferior turbinate. (**c**) Association between treatment and olfactory improvement; although not reaching significance, MCID $\Delta SIT \geq 4$ was observed in a subset of subjects over 4 months, trending towards an increase in the beclomethasone arm (Fischer's exact test, at 1 mo p=0.28, at 3 mo p=0.59), see **Table 2** for additional details.

Table 1. Demographic data for pilot controlled olfactory dysfunction trial

Group	Subject	Age	Sex	Prior therapy[a]	Parosmias	Anosmia onset	Treatment
Individual-level data							
	1	21	M	yes	yes	2020	Beclomethasone
	2	69	F	yes	yes	2021	Beclomethasone
	3	62	F	yes	yes	2022	Beclomethasone
	4	64	M	no	no	2022	Beclomethasone
	5	73	M	yes	yes	2020	Beclomethasone
	6	23	F	no	no	2020	Beclomethasone
	7	61	F	no	yes	2021	Beclomethasone
	8	24	F	yes	yes	2021	Placebo
	9	69	F	yes	yes	2020	Placebo
	10	60	F	yes	yes	2020	Placebo
	11	55	F	no	no	2022	Placebo
	12	68	F	yes	yes	2021	Placebo
	13	45	M	yes	no	2021	Placebo
	14	80	F	yes	no	2020	Placebo
	15	47	M	yes	no	2021	Placebo
Summary Characteristics							
	-	53.3	57.1%F	57.1%	71.4%	2020.9	Beclomethasone
	-	56.0	75.0%F	87.5%	50.0%	2020.8	Placebo

[a]Prior therapies included any of the following: oral steroid course, nasal steroid sprays, stellate ganglion nerve block, platelet rich plasma, olfactory training, and/or oral supplements.

Table 2. Improvement trend in olfaction (SIT score) at 1 and 3 months

Timepoint	Treatment	n	SIT (Mean, SEM)	% Improved	Absolute % Improved (95% CI)	Relative Improvement (95% CI)
1 month	Beclomethasone	6	23.8 (1.3)	66.67%	38.10% (2 to 97%)	2.14 (0.73 to 7.79)
	Control	7	20.7 (2.5)	28.57%	reference	reference
3 month	Beclomethasone	6	23.6 (3.0)	66.67%	23.81% (-17 to 80%)	1.74 (0.52 to 6.5)
	Control	7	23.1 (3.2)	42.86%	reference	reference

Comparisons of MCID in the Smell Identification Test score at one month and three months post treatment from baseline demonstrating absolute and relative improvements between treatment groups. SEM = Standard Error of the Mean

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Ed summary:

Kim, D'Anniballe et al. examined olfactory tissue from individuals with Long COVID-related anosmia and conducted a randomised pilot trial of topical steroid therapy. They found no persistent viral presence, identified clonally expanded inflammatory T cells, and confirmed the therapy's safety, supporting the need for larger trials.

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