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Single-cell RNA sequencing of mast cells in eosinophilic esophagitis reveals heterogeneity, local proliferation and activation that persists in remission

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Abstract

Background: Mast cells (MCs) are pleiotropic cells that accumulate in the esophagus of patients with eosinophilic esophagitis (EoE) and are thought to contribute to disease pathogenesis, yet their properties and functions in this organ are largely unknown.

Objective: We aimed to perform a comprehensive molecular and spatial characterization of esophageal MCs in EoE.

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Author's contribution

MER, NBBM, DFD, and NAB conceived of the study and study design. NBBM, TW, and TS performed the experiments and acquired the data. NBBM analyzed the data. NBBM, MER, DFD, and NAB established the analytical strategy. DFD, AB, and JMF provided bioinformatic assistance. JMC and KK coordinated human sample collection. VAM, PEP, and SMB provide the biopsies samples. NBBM prepared figures for the manuscript and wrote initial and subsequent drafts. MER, DFD, NAB, and JMC provided editing assistance. MER, DFD, and NAB supervised the study. All authors contributed to the interpretation of data and critically reviewed the manuscript for important intellectual content. All authors approved the final version of this manuscript.

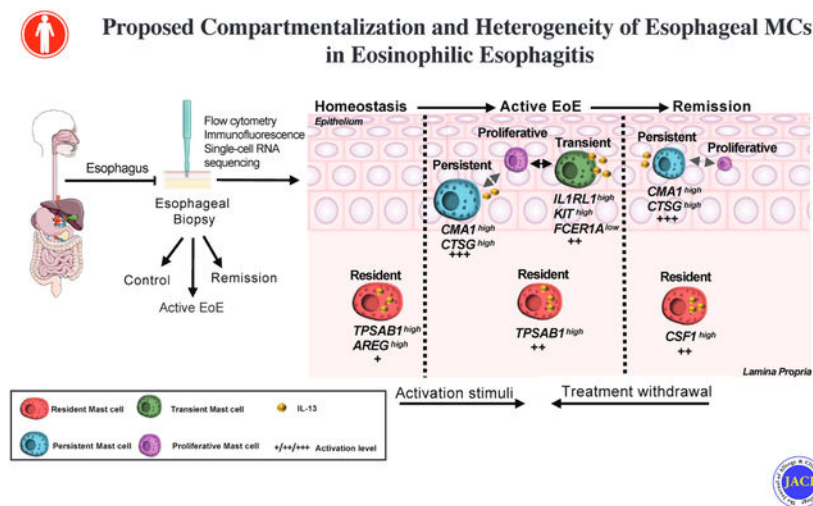
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Methods: Esophageal biopsies obtained from patients with active EoE, patients with EoE in histologic remission, and individuals with histologically normal esophageal biopsies and no history of esophageal disease (i.e., control individuals) were subject to scRNA-sequencing, flow cytometry, and immunofluorescence analyses.

Results: We probed 39,562 single esophageal cells by single-cell RNA sequencing; approximately 5% of these cells were MCs. We identified dynamic MC expansion across disease states. During homeostasis, *TPSAB1*^{high}*AREG*^{high} resident MCs were mainly detected in the lamina propria and exhibited a quiescent phenotype. In patients with active EoE, resident MCs assumed an activated phenotype, and two additional pro-inflammatory MC populations emerged in the intraepithelial compartment, each linked to a proliferating *MKI6*^{high} cluster. One pro-inflammatory activated MC population, marked as *KIT*^{high}*IL1RL1*^{high}*FCER1A*^{low}, was not detected in disease remission (termed transient MC), whereas the other population, marked as *CMA1*^{high}*CTSG*^{high}, was detected in disease remission where it maintained an activated state (termed persistent MC). MCs were prominent producers of esophageal IL-13 mRNA and protein, a key therapeutic target in EoE.

Conclusions: Esophageal MCs comprise heterogeneous populations with transcriptional signatures associated with distinct spatial compartmentalization and EoE disease status. In active EoE, they assume a pro-inflammatory state and locally proliferate, and remain activated and poised to re-initiate inflammation even during disease remission.

Graphical Abstract



Capsule summary:

We have performed a comprehensive examination of esophageal MCs across EoE diagnostic states assessing their expansion and activation profile by transcriptional and protein analytic methods, providing insight into their role in disease pathogenesis.

Keywords

Eosinophilic esophagitis; Mast cells; Single-cell RNA sequencing; IL-13; Proliferation

INTRODUCTION

Eosinophilic esophagitis (EoE) is a chronic, allergic, type 2 (T2) inflammatory disease driven by food antigens^{1, 2}. Histologic evaluation of eosinophil accumulation currently serves as the gold-standard diagnostic criteria for diagnosis and monitoring of disease activity. Active disease is defined as the presence of greater than or equal to 15 eosinophils per high-power field (HPF) in biopsy samples of the esophageal mucosa. The disease is clinically managed through dietary restrictions, anti-inflammatory treatment (e.g., topical glucocorticoids, proton pump inhibitors, biological treatment), and esophageal dilatation^{3, 4}. However, there is currently no cure nor FDA-approved treatment for EoE, and the disease typically follows a chronic, relapsing course⁵.

Genome-wide transcriptional profiling of the esophagus in patients with active EoE has revealed abundant expression of mast cell (MC)-associated genes, including those encoding tryptase (*TPSAB1*), carboxypeptidase A3 (*CPA3*), and c-KIT (*KIT*), implying MC accumulation⁶. Indeed, there is an accumulation of esophageal MCs in the epithelial layer but not in the lamina propria in patients with EoE⁷. Additionally, in patients with active EoE, MCs are degranulated, and their levels correlate with cardinal symptoms, such as dysphagia^{8, 9}. Furthermore, in patients with EoE in remission (defined by reduced esophageal eosinophil levels), increased MC concentrations are associated with persistent endoscopic and histologic abnormalities¹⁰. Additionally, increased esophageal *CPA3* and chymase (*CMA1*) expression are associated with a steroid-refractory endotype of EoE¹¹, and MC reduction is associated with clinical response to emerging biological therapies, including IL-5, IL-13, and Siglec-8 blockade¹²⁻¹⁴. Thus, tissue MC expansion and transcriptional activity are common in EoE and may be a key pathogenic contributor.

MCs are tissue-resident immune effector cells that exhibit context-dependent expression of pro-inflammatory mediators (e.g., proteases, cytokines) and receptors (e.g., activating, inhibiting, and growth factor receptors) and can demonstrate variable responses to pharmacologic inhibitors¹⁵. In homeostasis, MCs reside within barrier tissues, including the skin, airways, and gastrointestinal tract^{16, 17}. Tissue mastocytosis is a common feature of T2 inflammation that is thought to arise from the recruitment of rare, bone marrow-derived circulating MC progenitors^{8, 18, 19}. However, SCF can promote local proliferation of differentiated tissue mast cells^{20, 21}. Overall, the mechanisms directing MC hyperplasia in these settings are not well understood.

The two canonical subsets of human MCs are recognized on the basis of their protease expression. MCs containing tryptase, chymase, and CPA3, termed MC_{TC}, are commonly found in the skin and the intestinal submucosa. In contrast, MCs that express tryptase but not chymase, termed MC_T, are commonly found at mucosal surfaces²³⁻²⁴. Nuanced phenotyping of murine MCs has suggested that MC heterogeneity results from their plasticity and interaction with the microenvironment²⁵⁻²⁷. The transcriptomic profile of murine MCs isolated from multiple organs, including the trachea, esophagus, tongue, skin, and peritoneum, demonstrated wide transcriptional heterogeneity across the different tissues²⁸. Moreover, studies using single-cell RNA sequencing (scRNA-seq) demonstrate multiple, distinct MC subsets in the human intestine²⁹, lung³⁰, and nasal polyp³¹.

Herein, we conduct a comprehensive examination of esophageal MCs in EoE using scRNA-seq, flow cytometry, and immunofluorescence on esophageal biopsies obtained from patients with active EoE (active EoE), patients with EoE in histologic remission (remission), and individuals with histologically normal esophageal biopsies and no history of esophageal disease (i.e., control individuals). Through these approaches, we assess MC composition and transcriptional heterogeneity across the full spectrum of EoE and evaluate the potential contribution of MCs to both disease pathophysiology and relapse. We found that MCs in control individuals were present at relatively low levels (termed resident MCs). The esophageal MC pool expanded in individuals with active EoE due to the accumulation of two additional MC populations, each associated with local proliferation and activation. One of the EoE-specific MC populations waned with disease remission (termed transient MCs), whereas the other MC population persisted in individuals with EoE in remission (termed persistent MCs). We demonstrate that esophageal MCs further express disease-associated-transcriptional signatures, with the persistent population exhibiting an increased percentage of activation-associated genes even during disease remission. Notably, histologic and scRNA-seq analyses demonstrated that MCs are a prominent source for the key EoE driver cytokine IL-13 in all disease states. Collectively, the data presented herein show that the esophageal MC pool comprises dynamic populations that assume a pro-inflammatory phenotype in patients with active EoE and that specific MC populations (resident, persistent) remain transcriptionally activated and poised to re-initiate inflammation even during remission.

METHODS

Human subjects

Written informed consent was obtained before a patient's enrollment in the study, and all human studies were approved by the CCHMC Institutional Review Board (IRB #2008-0090). Control patients were defined as having no history of EoE diagnosis, having 0 eosinophils per 40X microscopic high-power field (HPF) of esophageal biopsy samples, and having no evidence of esophagitis within esophageal biopsies. Patients with active EoE had clinician-diagnosed EoE defined by a histologically active disease with ≥ 15 eosinophils in distal esophageal biopsies. Patients with inactive EoE (i.e., remission) had clinician-diagnosed EoE in the past and were defined by a histologically inactive disease with a peak eosinophil count of < 15 eosinophils per HPF in distal esophageal biopsies. An independent cohort of 10 patients was enrolled for the scRNA-seq analyses (control, $n = 2$; active EoE, $n = 5$; EoE remission, $n = 3$), an independent cohort of 13 patients was enrolled for the flow cytometry to assess mast cell levels (control, $n = 3$; active EoE, $n = 6$; EoE remission, $n = 4$), and an independent cohort of 20 patient biopsies was used for immunofluorescence of proteins (control, $n = 6$; active EoE, $n = 7$; EoE remission, $n = 7$) (Supplementary Table 1). A previously reported cohort of patients with active EoE ($n = 147$) analyzed by the EoE diagnostic panel^{11, 32} and an independent cohort with active EoE ($n = 10$) analyzed by bulk RNAseq⁶ were also analyzed in this study.

Enzymatic digestion of esophageal biopsies to produce a single-cell suspension

A single biopsy from the distal esophagus was collected into RPMI-1640 medium supplemented with 10% FBS, kept on ice, and transported to the research laboratory within 30 minutes for processing. The biopsy was then transferred into EDTA buffer (5 mM EDTA, 25 mM HEPES, 10% FBS in HBSS) for 15 minutes at 37°C, washed once with 1X phosphate-buffered saline (PBS) (pH 7.4), minced, and then subjected to collagenase A (2.4 mg/mL, Roche) digestion for 30 minutes at 37°C. The resulting suspension was diluted with ice-cold PBS, passed through a 19-gauge needle, filtered through a 70-µm cell strainer, and washed with ice-cold PBS.

Flow cytometry staining

A single-cell suspension, generated by enzymatic digestions, was pelleted in PBS before viability staining (Invitrogen L34957, 1:1000) for 10 minutes; cells were washed with 2% FBS in PBS for antibody staining. Cells were incubated with Fc Block (BD Biosciences 564219, 1:50) for 15 minutes before adding labeled antibodies. Cells were stained for CD45 (BV711, Biolegend 103147, 1:50), FcεR1α (FITC, Biolegend 334608, 1:50), and c-KIT (PE, Biolegend 313204, 1:50) for 30 minutes at 4°C. Samples were then resuspended in PBS + 2% FBS and analyzed on a Fortessa cytometer in the Cincinnati Children's Hospital Medical Center (CCHMC) Research Flow Cytometry Core (RFCC). Single-color compensation was calculated, and data were analyzed in FlowJo (Version 10.6.1).

Immunofluorescence staining

Distal esophageal biopsies were fixed with 10% formalin and embedded in paraffin (FFPE). FFPE samples were sectioned and de-paraffinized using xylene and then subjected to graded ethanol washes. Heat-induced epitope retrieval in R-UNIVERSAL Epitope Recovery Buffer (EMS, 62719–20) was performed. Slides were blocked in PBS with 10% donkey serum for 1 hour followed by overnight incubation at 4°C in the following primary antibodies: mouse anti-human tryptase (Biolegend, 369402), rabbit anti-human KI-67 (Invitrogen, PA-19462), or rat anti-human IL-13 (Biolegend, 501902). Slides were then washed, incubated for 1.5 hours at room temperature with Hoechst 33342 nuclear staining (Invitrogen, H3570) and the following secondary antibodies: donkey anti-mouse Alexa Fluor 647 (Jackson, 715605151), donkey anti-rat Alexa Fluor 488 (Invitrogen, A21208), or donkey-anti-rabbit Alexa Fluor 488 (Jackson, 711545152). Then slides were washed and mounted with ProLong™ gold antifade reagent (Invitrogen, P10144). Images were obtained using the NIKON A1 inverted LUNV confocal microscope. Quantifying confocal sections were carried out with Imaris and Elements software.

Single-cell RNA sequencing analysis

Single-cell suspensions were prepared from biopsies as described above. Bulk population cells were directly subjected to the 10X mass genomics chip (10X Genomics, Inc.), targeting 10,000 simultaneously captured live events for next-generation sequencing. Each cell was uniquely barcoded during the cDNA library generation, subsequently sequenced on an Illumina HiSeq 2500 at CCHMC's DNA sequencing core, allocating a total read of ~320M (2 lanes/flow cell). Sparse data matrices, provided by 10X genomics, were used as input

into Seurat analyzed in R studio (R version 3.6.1) as previously described^{33, 34}. For analysis of all sequenced esophageal samples, cells were filtered on the basis of unique feature counts of over 200 or less than 4,800, with less than 20% mitochondrial counts. In addition, genes that were expressed in fewer than three cells were excluded. In sum, 39,562 cells and 20,208 genes passed these filter criteria. A gene's relative expression was calculated by log normalization and centering. Principal component analysis (PCA) was performed with the list of the top 2000 variable genes. Data were subjected to Uniform Manifold Approximation and Projection (UMAP) and shared nearest neighbor (SNN) modularity optimization-based clustering, identifying 21 distinct clusters using 12 dimensions and 0.8 resolution parameters. Top marker genes with high specificity were used to classify cell markers into seven known cell types: epithelium, fibroblast, endothelium, T cells, B cells, myeloid cells, and MCs. The MC population, a total of 1,897 cells, was subject to subsetting and re-PCA and SNN modularity optimization-based clustering, which identified six distinct clusters using 4 dimensions and 0.3 resolution parameters. They were classified and merged into 4 distinct populations on the basis of their differential presence: resident, transient, persistent, and proliferative populations.

Non-epithelial clusters identified as strongly expressed marker genes associated with esophageal epithelium were considered contaminated and discarded. PCA and SNN modularity optimization-based clustering steps were performed before the beginning of the analysis.

Gene enrichments were calculated using the FindAllMarkers function in Seurat. The Wilcoxon test was implemented in Seurat with the default parameter threshold set of 0.25 unless mentioned otherwise to identify cluster-defined genes.

MST pseudotime analysis

A minimum spanning tree (MST) map was created in R by first extracting the cell data and cluster metadata from the Seurat object. Using the dyno package³⁵, as previously described at <https://github.com/dynverse/dyno>, the cell data were competently converted to a wrapper object containing the cell expression, counts, and cluster metadata and compatible with the dyno package. Then, an MST trajectory was inferred and plotted using the dyno package. This trajectory was then added back to the original Seurat object for further visualization by grouping the cells onto their nearest milestone and adding these data to the Seurat object.

Gene ontology (GO) analysis

GO analysis was performed using the ToppGene website (<https://toppgene.cchmc.org/>)

Statistical analyses

Statistical significance was determined for normal distribution using one-way ANOVA with Tukey's multiple comparisons post-test or a Student's t-test (unpaired, two-tailed). For non-normal distribution, Mann-Whitney test or Kruskal-Wallis's test with Dunn's multiple comparison post-test were used. Correlation analysis was performed using Spearman's rank correlation coefficient, followed by Bonferroni-Dunn's adjustment. Statistical analyses were

performed using GraphPad Prism (version 8.0.1, GraphPad Software Incorporated, La Jolla, CA, USA)

RESULTS

Esophageal MCs increase in human active EoE, persist during remission, and strongly associate with the EoE cytokine milieu

Flow cytometry analysis of a cohort of human esophageal biopsies (Supplementary Table 1), including control (n = 3), active EoE (n = 6), and remission (n = 4), identified FcεR1α^{high}-KIT^{high} MCs in the tissue (Figure 1A). MCs were a highly prevalent white blood cell during active EoE, accounting for 22 ± 7.5% of total white blood cells in patients with active EoE compared to 4.2 ± 1.8% in control individuals (Figure 1B). Notably, although a trend of decreased MC levels was observed in remission biopsies compared to active EoE, MCs remained elevated in disease remission compared to control, accounting for 11.7 ± 4.1% of total white blood cells (Figure 1B). This finding contrasts with that of eosinophils which were identified as FcεR1α^{intc}-KIT^{int} FSC^{high}SSC^{high} 36 (Figure 1A); eosinophils increased to 18.7 ± 11.1% of total white blood cells in active EoE compared to 0% in control esophageal tissue but were nearly fully depleted in remission biopsies (Figure 1A–B). Eosinophil levels were confirmed by histologic quantification of the tissue sections and accounted for 0 eosinophils/HPF in control, 46 ± 14 eosinophils/HPF in active EoE, and 1 ± 2 eosinophils/HPF in remission (Supplementary Figure S1).

To determine the relationship between MC concentration and EoE-related inflammatory mediators, we examined bulk RNA sequencing data from the esophageal tissue of patients with active EoE (n = 10)^{6, 93}. In active disease, the MC marker genes *TPSAB1* and *CPA3*⁸ had a high, positive correlation with the EoE-associated cytokines *IL13* (Spearman r = 0.88, P = 1.3E-2; and Spearman r = 0.90, P = 6.4E-3, respectively), *IL5* (Spearman r = 0.90, P = 6.4E-3 and Spearman r = 0.92, P = 4.0E-3, respectively). (Figure 1C–F)³⁷. These data suggested the potential importance of MCs as a source of several major components of the inflammatory milieu in active EoE.

scRNA-seq: esophageal MCs are poised to generate pro-inflammatory mediators

To further assess the potential contribution of MCs to EoE pathobiology relative to other cell populations, we conducted scRNA-seq on 39,562 single cells from esophageal biopsies (n = 5 patients with active EoE; n = 3 patients with EoE in remission, and n = 2 non-EoE controls) (Supplementary Table 1). Seven major cell lineages were identified on the basis of hallmark gene expression (shown in parentheses): epithelial cells (*KRT13* and *KRT5*), T cells (*CD3E* and *CD7*), endothelial cells (*SPARC* and *VWF*), myeloid cells (*HLA-DRB* and *HLA-DRA*), fibroblasts (*DCN* and *COL1A1*), B cells (*MS4A1* and *CD79A*), and MCs (*TPASB1*, *CPA3*, *GATA2*, and *MITF*) (Figure 2A–B and Supplementary Table S2). Although eosinophils were present in histologic samples (Supplementary Figure S2A), no cluster was detected expressing the eosinophil marker transcript *CLC*. This finding is consistent with prior scRNA-seq studies of respiratory tissue^{38, 39} that failed to detect eosinophils, likely due to their high expression of RNases, such as eosinophil cationic protein (*RNASE3*) and eosinophil-derived neurotoxin (*RNASE2*)⁴⁰. MCs were a

prominent cell type in the esophagus, comprising $7.3 \pm 2.4\%$ of total recovered cells during active disease (Supplementary Figure S2B). In active EoE patients, in addition to the enriched expression of the hallmark proteases *TPSAB1*, *CPA3*, *CTSG*, and *CMA1*, the MC cluster exhibited enrichment of the cathepsin family proteases *CTSD* and *CTSW*; however, the expression of these genes was not exclusively restricted to MCs (Figure 2C and Supplementary Table S2). MCs were also the most prevalent population that expressed genes associated with histamine biosynthesis (*HDC*), prostaglandin biosynthesis (*HPGDS*, *PTGS1*, and *PTGS2*), and leukotriene biosynthesis (*LTC4S*, *ALOX5AP*, and *ALOX5*) (Figure 2C and Supplementary Table S2). MCs together with T cells significantly expressed the type 2 cytokine–encoding genes *IL5* and *IL13* and colony-stimulating factor 1 (*CSF1*, aka macrophage-colony stimulating factor 1) (Figure 2C and Supplementary Table S2). MCs were also the only cells that significantly expressed the IL-6 cytokine family member leukemia inhibitory factor (*LIF*) (Figure 2C and Supplementary Table S2). MCs further expressed mRNA encoding diverse cytokines, chemokines, and growth factors, including *CCL2*, *CCL4*, *TGFB1*, *AREG* (encoding Amphiregulin), and *VEGFA*, although expression of these genes was not restricted to MCs (Figure 2C and Supplementary Table S2). MCs were also the main esophageal population expressing the receptors for IL-33 (*IL1RL1*) and c-KIT (*KIT*), expressed the highest level of the aryl hydrocarbon receptor (*AHR*), and were the only esophageal cell population that expressed all three subunits of the high-affinity IgE receptor FcεR1 (i.e., *FCER1A*, *FCER1G*, and *MS4A2*) (Figure 2C).

scRNA-seq: MCs are engaged in multiple cellular axes of the esophageal ecosystem

We next directly evaluated MCs within the context of the cellular signaling axes thought to promote inflammation in EoE. We identified cellular expression of key type 2 inflammation–associated cytokines and the corresponding receptors within the scRNA-seq dataset, identifying potential interactions between MCs and the other detected esophageal populations, including myeloid cells, fibroblasts, endothelial cells, and epithelial cells (Supplementary Figure 3A–C). The MC survival factor *KITLG* was expressed by structural cells, including fibroblasts, endothelial, and epithelial cells; the latter cell demonstrated increased expression in active EoE (Figure 3A–B and Supplementary Figure 3A–C)^{41, 42}. In addition, MC expression of *IL1RL1* indicated a possible networking with cells expressing *IL33*, a cytokine that can promote human MC activation, survival, and adhesion⁴³. Interestingly, although *IL33* was mainly detected in endothelial cells (Figure 3C–D), during active EoE, epithelial cells exhibited increased *IL33* expression. MC's *IL13* expression was predominantly observed in active EoE (Figure 3E–F), supporting the correlation between *TPSAB1* and *IL13* observed in bulk sequencing of EoE biopsies (Figure 1C and Figure 1E).

In addition, in control biopsies, MCs expressed *AREG*, indicating a potential communication axis with *EGFR*+ epithelial cells (Supplementary Figure 3A), an interaction that was previously demonstrated as essential for normal epithelial differentiation^{44, 45}.

In active EoE, MCs expressed several additional inflammatory mediators, including *IL5*, potentially allowing them to interact with eosinophils, and *CSF1*, potentially allowing them to interact with *CSF1R*-expressing myeloid cells, an essential interaction for myeloid differentiation and expansion^{46, 47} (Supplementary Figure 3B). MCs also expressed *TGFB1*,

suggesting interaction with *TGFBR2*-expressing fibroblasts, which has the potential to promote fibroblast proliferation and collagen production⁴⁸ and contribute to the Mendelian-inherited form of EoE caused by *TGFBR2* mutations (Supplementary Figure 3B)⁴⁹. MCs also expressed elevated *LIF*, allowing possible interactions with endothelial cells that express *LIFR*, a receptor that regulates angiogenesis^{50–52}. Finally, MCs expressed *VEGFA*, congruent with a previous report which demonstrated that activated MCs secrete VEGFA⁵³, suggesting an additional axis of communication with *FLT1+* endothelial cells, an interaction previously shown to promote endothelial differentiation⁵⁴ (Supplementary Figure 3B). Interestingly, many potential interactions established in active disease persisted in remission biopsies (Supplementary Figure 3C).

We also evaluated the cellular expression of transcripts encoding lipid mediators and histamine biosynthetic enzymes along with the corresponding receptors. MCs expressed the leukotriene-associated genes *LTC4S*, *ALOX5AP*, and *ALOX5*, indicating potential interactions with *CYSLTR1+* endothelial cells and *CYSLTR1+ CYSLTR2+* fibroblasts, all of which could be observed across disease states. These interactions have been shown to promote inflammatory signals and proliferation in endothelial cells and fibroblasts^{55, 56}. MCs expressed the histamine biosynthesis-associated gene *HDC*, indicating the potential to interact with esophageal *HRH1+* endothelial cells across disease states. In contrast, myeloid cell expression of *HRH2* was only observed in active EoE. MC expression of the prostaglandin biosynthesis-associated genes *HPGDS*, *PTGS1*, and *PTGS2* indicated the potential to associate with *PTGDR2+* fibroblasts. This interaction has been shown to promote fibroblast activation and is associated with fibrosis⁵⁷ (Supplementary Figure 3D–F).

scRNA-seq: increased MC heterogeneity during EoE, including an actively proliferating MC population

After re-clustering 1,897 single-cell MC transcriptomes, scRNA-seq analysis identified six MC clusters (Supplementary Figure S4A). Three of them were present in biopsies from all groups, termed resident MC 1–3 (Supplementary Figure S4B–D). To overcome the limitation of MCs numbers in control biopsies and as pooling resident MC 1–3 populations mostly maintained the expression levels of hallmark proteases, lipids, cytokines, and receptors compared to the other populations across the disease and control samples (Supplementary Figure S4E), these populations were pooled for subsequent downstream analysis and termed “resident MCs” (Figure 4A). The revised clustering was conserved across multiple donors (Supplementary Figure S5A–B). The resident population accounted for $100 \pm 0\%$ of control MCs, $42 \pm 27\%$ of active EoE MCs, and $83 \pm 22\%$ of remission MCs (Figure 4B). Further analysis identified three additional MC populations in patients with active EoE. The first was designated transient MCs, as it was only detected in active disease, accounting for $19 \pm 22\%$ of esophageal MCs (Figure 4B). The second EoE-associated population was designated persistent MCs, as it was observed in both active EoE (accounting for $37 \pm 24\%$ of total MCs in active EoE) and in remission (accounting for $17 \pm 21\%$ of the total MCs during remission) (Figure 4B). The third EoE-associated MC cluster was designated as proliferative MCs on the basis of enriched expression of proliferation-associated genes, including *MKI67*, *TOP2A*, *PCNA*, and *KIAA0101* (aka PCLAF, PCNA clamp-associated factor⁵⁸) (Figure 4C and Supplementary Table S3). GO

analysis corroborated enriched expression of genes involved in the cell cycle (corrected p-value = 3.8E-70) and cell division (corrected p-value = 1.9E-56) within the proliferative MC cluster (Supplementary Table S4).

Immunofluorescence analysis of biopsies from an independent patient cohort (control, n = 6; active EoE, n = 7; remission, n = 7) (Supplementary Table 1) revealed that MCs in control biopsies were located mainly in the lamina propria and the level of this population did not significantly change in active EoE or remission (Supplementary Figure S6A, Supplementary Figure 6C–D, and Supplementary Figure 6F). As control samples were only found to contain resident MCs present by scRNA-seq analysis (Figure 4B), we concluded that resident MCs are likely localized in the lamina propria. MC numbers were significantly increased in the epithelium layer, but not in the lamina propria, in active EoE compared with control biopsies (Supplementary Figure S6A–B), consistent with previous reports⁷. Biopsies obtained from patients with EoE in remission exhibited variability in the intraepithelial MC levels (Supplementary Figure S6D–F), consistent with a recent report¹⁰.

The proliferative cluster was predominantly observed in active EoE biopsies, accounting for $2.0 \pm 2.7\%$ of total MCs (Figure 4B). Immunofluorescent staining of an orthogonal patient cohort (control, n = 3; active EoE, n = 5; remission, n = 6) (Supplementary Table 1) confirmed the presence of KI67+ MCs within the esophagus during active and remission (Figure 4D–E).

The MC clusters were further analyzed through pseudotime using independent Minimum Spanning Tree (MST) trajectory analysis^{59, 60} (Figure 4F). The proliferative population was distributed between both transient and persistent MCs through this approach, suggesting that the increased MC proliferation observed histologically during EoE (Figure 4D–E) could be attributed to these populations (Figure 4F). Indeed, quantification of KI67+ MCs in each compartment in biopsies obtained from patients with active EoE, in which all populations were present, demonstrated that proliferation occurs mainly within the epithelium (Figure 4G). The MST trajectory analysis demonstrated that the proliferating cells were observed in the persistent population during remission; however, the number of cells was too low to be defined accordingly (n = 2) (Supplementary Figure S7).

Marker gene analysis identified disease state-dependent MC transcriptional signatures

To further examine the transcriptional differences in esophageal MCs across disease states, we evaluated differential gene expression across both the entire MC population as a whole and within each subpopulation. This analysis demonstrated that MCs obtained from control biopsies were composed of resident MCs which, compared with MCs that were obtained from active EoE and remission biopsies, exhibited increased expression of *TPSAB1*, *ALOX5*, and *AREG*; the latter encodes for a protein that maintains epithelial homeostasis^{44, 45} (Figure 5A and Supplementary Table S5). MCs from active EoE were enriched in the expression of the protease-encoding transcripts *CPA3* and *CTSG*; the lipid mediator biosynthetic enzyme-encoding transcripts *ALOX5AP* and *PTGS2*; the cytokine, chemokine, and growth factor-encoding transcripts *CCL2*, *VEGFA*, *IL5*, and *IL13* and the receptor-encoding transcripts *IL1RL1*, *FCER1A*, *AHR*, *CSF2RB*, *MS4A2*, and *KIT*, compared with MCs derived from control and remission biopsies (Figure 5A and

Supplementary Table S5). Next, we were interested in evaluating if the higher level of mRNA transcripts in MCs obtained from active EoE patients also translated to an increased protein level. As a proof of principle, we evaluated FcεR1A protein levels. We found that MCs from active EoE patients exhibited higher FcεR1A levels compared with MCs from control and remission (Supplementary Figure S8), consistent with *FCER1A* mRNA levels. Subset analysis (Figure 5B) indicated that resident MCs were most enriched for expression of *IL5*, transient MCs were enriched for expression of the receptor-encoding genes *IL1RL1* and *KIT* and expressed the lowest level of *FCER1A*, and persistent MCs were enriched for expression of *CTSG* and *CMA1*, suggesting an MC_{TC} phenotype (Supplementary Table 6). In biopsies obtained from patients with EoE in remission, MCs as a whole exhibited the highest enrichment of the protease-encoding transcripts *CTSD*, *CTSW*, and *CMA1*; lipid mediator-associated genes *LTC4S*, *HPGDS*, *HDC*, and *PTGSI*; the cytokine and growth factor-encoding transcripts *LIF*, *TGFB1*, and *CSFI*; and the signaling adaptor-encoding transcript *FCER1G* compared with that of MCs identified in control and active EoE biopsies (Figure 5A and Supplementary Table S5). In remission, subset analysis indicated that the resident MC population was most enriched for *CSFI*. The persistent population exhibited enrichment of the protease-encoding transcripts *CMA1* and *CTSG* (Figure 5B and Supplementary Table S6).

MC activation signatures indicate transcriptional activation during active disease and remission

The transcriptional signature of MCs across disease activity states (Figure 5C) suggested that all MC populations, including the resident population, upregulated inflammatory genes during active EoE and remission, consistent with a heightened activation state. We thus examined the expression of MC activation genes of the total MCs transcriptome across the different disease states (Supplementary Table S8)⁶¹. MCs from active EoE and remission biopsies were more activated than those derived from control biopsies (Figure 5D). Notably, despite differences in transcripts encoding pro-inflammatory mediators observed between active EoE and remission (Figure 5A), no difference was found in total MC activation signature expression between active EoE and remission (Figure 5C). Subset analysis indicated that the resident MC population exhibited an increased activation signature in both active EoE and remission compared with non-EoE controls (Figure 5D). Specific analysis of active EoE, the only disease state in which all three major populations could be observed, indicated that the persistent population exhibited the highest overall activation signature expression (Figure 5E). No difference in activation signature expression was observed in persistent MCs between active EoE and remission (Figure 5F). Relative to the other MC populations, persistent MCs exhibited increased expression of 307 unique genes and decreased expression of only 17 genes. GO analysis of biological processes of the 307 elevated genes found enrichment in genes associated with an activation process (corrected p-value = 2.7E-25) and immune effector process (corrected p-value = 1.2E-22) (Supplementary Table S3 and Supplementary Table S7). Thus, the resident and persistent populations maintained the increased activation signature despite disease remission.

MCs were a prominent source of esophageal IL-13

IL13 was enriched in MCs compared with other esophageal cells in active EoE biopsies (Figure 2C) and undetectable in control biopsies (Figure 5A and Supplementary Figure S9A) and correlated with MC protease expression during active EoE in bulk RNAseq analysis (Figure 1C and Figure 1E). Using immunofluorescence, we evaluated IL-13 protein content in MCs from control (n = 6) and active EoE (n = 7) (Supplementary Table 1). In control biopsies, although resident MCs did not express detectable *IL13* mRNA (Figure 5A and Supplementary Figure S9A), they contained a readily detectable intracellular IL-13 (Figure 6A, top row). In active EoE, resident MCs upregulated their *IL13* transcripts (Figure 5B) and IL-13+ MC numbers in the lamina propria were unchanged with 214 ± 117 IL-13+ MCs/mm² lamina propria in control samples and 263 ± 143 IL-13+ MCs/mm² lamina propria in active EoE (Figure 6B). During active EoE, although most MCs within the epithelium lacked intracellular IL-13 (e.g., $20 \pm 9\%$ were IL-13+), the concentration of IL-13+ intraepithelial MC numbers increased significantly to 40 ± 18 MCs/mm² epithelium in active EoE compared with 12.5 ± 5 MCs/mm² epithelium in controls, consistent with the enrichment of *IL13* observed in the transient and persistent intraepithelial populations during active EoE (Figure 6A–B and Figure 5A–B). The high degree of activation observed in all MCs in active EoE (Figure 5E) coupled with the accumulation of IL13+ MCs in the intraepithelial compartment, and MC's ability to secrete stored intracellular cytokines upon activation^{62, 63} suggests that the activated intraepithelial MCs may actively secrete IL-13 during disease. This possibility is supported by the positive correlation of *TPSAB1*, a reliable marker of MC tissue burden⁸, with canonical IL-13 induced epithelial genes including *CAPN14*, *CCL26*, *CDH26* and *POSTN* (n = 147 patients with active EoE in an independent cohort)^{11, 32} (Supplementary Figure S9B–E).

DISCUSSION

MCs have well-described roles in type 2 diseases associated with IgE-mediated degranulation, such as urticaria and anaphylaxis⁶⁴. However, therapeutic targeting of IgE was largely unsuccessful in EoE^{65–67}. Herein, we demonstrate that MCs infiltrate the inflamed esophagus in EoE, exist in at least three distinct populations, proliferate locally, remain activated in disease remission, and generate IL-13, a primary mediator and therapeutic target in EoE^{68, 69}. In addition, the MC markers *TPSAB1* and *CPA3* are highly associated with type 2 cytokine-induced genes in the esophageal epithelium. These data highlight the importance of human MC effector function beyond IgE-mediated degranulation and suggest that control of tissue MC expansion will be required for complete remission of EoE.

We have found that MCs are present in the healthy esophagus and become highly prevalent cell type detected in active EoE. Furthermore, we find that specific MC subpopulations can remain elevated despite disease remission. We have established that MCs are present mainly in the esophageal lamina propria during homeostatic conditions, characterized as *TPSAB1*^{high}*AREG*^{high}, expressing a low level of MC activation-associated genes but can be readily activated as they store inflammatory proteins such as IL-13. In active EoE, MCs expand and infiltrate into the esophageal epithelium and proliferate locally.

They are characterized by the accumulation of *KIT*^{high}*IL1RL1*^{high}*FCER1A*^{low} transient and *CMA1*^{high}*CTSG*^{high} persistent EoE-specific MCs populations, each of which is associated with the *MKI67*^{high} proliferative cluster. In remission, the transient population is abolished, consistent with the decreased level of the intraepithelial MCs; however, the *CMA1*^{high}*CTSG*^{high} persistent population, in some patients, remains detectable in the intraepithelial compartment. Both *CSF1*^{high} resident and *CMA1*^{high}*CTSG*^{high} persistent populations remain transcriptionally activated even in disease remission. Supplementary Figure 10 summarizes these primary findings.

MC accumulation is a central feature of EoE^{7, 8} and is linked to multiple disease pathologies¹⁰. Thus, understanding their tissue accumulation is essential. Until now, evidence has demonstrated that MCs in the intestinal mucosa arise from bone marrow-derived progenitors that constitutively home to the intestine during T cell-mediated inflammation^{70–72}. We report that in biopsies obtained from patients with EoE, MCs exhibit increased local proliferation in the esophagus associated with the increased proliferation of the transient and the persistent MC populations. Increased local proliferation during active EoE is aligned with a recent paper that reported local MC proliferation in another type 2 inflammatory disease, chronic rhinosinusitis with nasal polyposis³¹. Therefore, MC expansion by proliferation is likely a common hallmark feature of type 2 diseases. Several factors upregulated in the esophagus in active EoE are candidates for inducing local MC proliferation. This includes stem cell factor (SCF), which is a prominent candidate as human and rodent MCs cultured with SCF undergo extensive proliferation ex vivo and in vivo, respectively^{22, 20}. Our data demonstrate the SCF-encoding gene, *KITLG*, is expressed mainly by fibroblasts and present in all EoE disease states although it is upregulated in epithelial cells in active disease (Figure 3A–B and Supplementary Figure 3A–C). In addition, the receptor *KIT* is upregulated in active EoE, especially by the transient population that is linked to MC proliferation (Figure 2C, Figure 3A, Figure 5A–B, and Supplementary Figures 3A–C). Another potential regulator of MC proliferation is IL-9, which also promotes MC maturation and activation^{12, 73, 74}. *IL9R* is enriched in MCs during active EoE, and remission compared with MCs obtained from control biopsies (Figure 5A–B and Supplementary Table S5). *IL9* is absent from the scRNA-seq dataset, perhaps due to the technological limitations (e.g., the absence of eosinophil gene signatures) or because it is produced by relatively rare cell populations (e.g., ILC2 and TH9 cells)^{73, 75}. Understanding the mechanisms that promote the local proliferation of MCs and whether they are specific to the esophagus or common to other type 2 diseases remains largely unexplored.

In homeostasis, MCs localize around the vasculature and nerves, and their wide range of receptors led to the notion that they serve as tissue sentinels. As MCs are pre-armed with an arsenal of proteases and inflammatory proteins, they have the potential to be one of the first responders to local stimuli, promoting acute local inflammation¹⁵. In control biopsies, MCs reside in the lamina propria and are characterized by the expression of proteases, such as chymase, which is known to increase epithelial permeability in the intestine^{7, 76}. During homeostasis, MCs contain a large amount of IL-13 protein (Figure 6A–B). This cytokine has potential to promote key inflammatory processes in EoE, such as activating the epithelium to secrete CCL26, inducing tissue remodeling, and impairing epithelial differentiation and barrier function³⁷. Although MCs are known to generate IL-13 in response to various

stimuli^{77, 78}, Th2 lymphocytes are thought to be the primary source of IL-13 in EoE. Th2 lymphocytes are not present in the esophagus under homeostatic conditions but are instead present in the tissue only during active EoE and remission⁸³. In addition, evidence is accumulating that Th2 lymphocytes may be clonal and home to the esophagus via GPR15 during active EoE^{84, 85}. Moreover, in active disease, we found that MC express abundant IL-13 protein and there was a high correlation between MC marker genes and expression of IL-13–elicited genes in bulk RNA-seq (Figure 6A–B and Supplementary Figure 9B–E), indicating that MCs also contribute to IL-13 generation in disease. Indeed, MCs are an essential source of IL-13 in other T2 inflammatory diseases, such as asthma⁷⁸ and nasal polyposis³¹. Other potential sources for esophageal IL-13 during active EoE are eosinophils, basophils and ILC2^{79, 80, 86, 87}. While eosinophils, similar to Th2, are present only during active disease, basophils and ILC2 are present in the esophagus also under homeostatic conditions^{36, 81, 82}. However, only basophils, similar to MCs, can store preformed IL-13 as ILC2 require T2 stimuli to generate and secrete IL-13^{81, 82, 86, 87}.

EoE exhibits defined histopathology, including local type 2 inflammation, angiogenesis, epithelial barrier disruption, epithelial hyperplasia, and fibrosis⁸⁸. During active disease, MCs expand in the epithelial compartment⁷ (Supplementary Figure S6A–C) and exhibit an enriched transcriptional activation signature in both the lamina propria and epithelial compartments (Figure 5). Our data suggest that their expansion allows them to interact with a wide range of esophageal cell populations, including epithelial cells (e.g., via IL-13–induced CCL26), eosinophils (e.g., via IL-5–induced eosinophil activation and survival), other myeloid cells (e.g., via CSF-induced dendritic cell activation), endothelial cells (e.g., via LIF and leukotriene-induced angiogenesis), and fibroblasts (e.g., via TGFβ1-induced proliferation) (Supplementary Figure 3)^{48, 52, 56, 89–91}. These findings provide rationale for therapeutic targeting of MCs in EoE; this is a timely finding as MC-depleting and/or MC-inhibiting drugs, such as anti-KIT (CDX-0159) and anti-Siglec-8 (lirentelimab), respectively, are in clinical testing^{14, 92}.

A recent study demonstrated that patients with EoE in histologic remission (i.e., less than 15 eosinophils/HPF) exhibited variability in histologic and clinical remission positively associated with MC levels¹⁰. In patients who exhibited persistent endoscopic and histologic abnormalities, MC numbers were elevated. Conversely, in patients showing complete histologic and clinical remission (i.e., no symptoms nor endoscopic abnormalities), MC numbers were decreased¹⁰. Our findings add increased resolution about the dynamic level and function of MCs in EoE by identifying a persistent population of activated MCs during histologic remission (defined by eosinophil levels) (Figure 4A–B, Figure 5B, and Figure 5E–F). The presented findings emphasize that MC resident and persistent subpopulations are activated in remission and poised to promote inflammation and that increased MC transcript expression is associated with increased IL-13 stimulation (Figures 5A–D and Figure 5F). Thus, we suggest that assessing MC burden may be a needed component in defining EoE remission.

Chymase and cathepsin G are classically associated with submucosal MC_{TC}^{22–24}. In control biopsies, resident MCs were primarily located in the lamina propria compartment and exhibited an MC_{TC} phenotype⁷. Our study identifies the *CMA*^{high}*CTSG*^{high} persistent

population located in the intraepithelial compartment in active disease and remission (Figure 5B, Supplementary Figure S6, and Supplementary Table S3). This finding is consistent with a previous study that demonstrated that during active EoE, MC_{TC} are also found in the intraepithelial compartment⁷. As the differences between mRNA and protein levels may exist due to storage of proteins in MC granules⁹⁴, further study will be required to determine whether the persistent intraepithelial MCs exhibit a MC_{TC} phenotype.

Our data demonstrate increased variation in the proportion of resident MC subpopulation between donors (Supplementary Figure 4D). These finding may result from differences in disease severity that influence the proportion of EoE-associated MC populations. Alternatively, variation in the size of the lamina propria in each biopsy may contribute. As resident MCs are localized in the lamina propria, the proportion of the resident MC is likely dependent upon the size of the lamina propria in each biopsy. To overcome this limitation, computational analysis verified that MCs subpopulations do not derive from donor variation but are rather dependent upon disease status. In addition, we combined resident subpopulations 1–3 to enable focus on diagnostic-dependent differences (Figure 4 and Supplementary Figure 5). To evaluate the possibility of the existence of multiple subpopulations in the resident MC population, a larger cohort will be examined in the future.

In conclusion, we have identified three populations of esophageal MCs that exhibit heterogenous properties defined by their (1) selective accumulation at different stages of disease and health; (2) expression of inflammatory genes; (3) level of transcriptional activation; (4) association with local proliferation; and (5) spatial compartmentalization. We demonstrate that MCs constitute a prominent source of key EoE mediators (e.g., IL-13) even under homeostatic conditions. In particular, during homeostasis, *TPSAB1*^{high}*AREG*^{high} resident MCs are mainly present in the lamina propria and exhibit a quiescent phenotype. In patients with active EoE, resident MCs assume an activated phenotype, and two additional pro-inflammatory MC populations emerge in the intraepithelial compartment, each linked to a proliferating *MKI6*^{high} cluster. The transient MC population, marked as *KIT*^{high}*IL1RL1*^{high}*FCERIA*^{low}, is not detected in disease remission, whereas the persistent MC population is marked by *CMA1*^{high}*CTSG*^{high} expression and persists even in disease remission. The finding that MC subpopulations remain present and primed for activation even in patients without esophageal eosinophilia provides a paradigm shift in defining disease remission as the mere absence of eosinophils and suggests that eosinophil levels may not be a sufficient marker of disease activity. Our data substantiate the importance of considering the presence of MCs, especially the persistent *CMA1*^{high}*CTSG*^{high} MC population identified herein. Thus, restraining different MC populations and their activity should be a therapeutic consideration for EoE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Competing Interests:

M.E.R. is a consultant for Pulm One, Spoon Guru, ClostraBio, Serpin Pharm, Allakos, Celldex Therapeutics, Celgene, Astra Zeneca, Adare/Elodi Pharma, GlaxoSmith Kline, Regeneron/Sanofi, Revolo Biotherapeutics, and Guidepoint and has an equity interest in the first five listed, and royalties from reslizumab (Teva Pharmaceuticals), PEESV2 (Mapi Research Trust) and UpToDate. M.E.R. is an inventor of patents owned by Cincinnati Children's Hospital. T.S. has received research support from JSPS Overseas Research Fellowships and is a co-inventor of patents owned by Cincinnati Children's Hospital Medical Center. V.A.M is a consultant for Takeda and Allakos.

All other authors declare that they have no competing interests.

Abbreviations:

EoE	Eosinophilic Esophagitis
MC	Mast cell
scRNA-seq	Single-cell RNA sequencing
T2	Type 2
HPF	High Power Field

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Key messages:

- Esophageal MCs are a dynamic population that can increase their numbers, heterogeneity, activation, and compartmentalization in active EoE and remain activated in remission.
- Esophageal MCs expand by in situ proliferation.
- Esophageal MCs constitute a prominent cellular source of inflammatory mediators (e.g., IL-13) even under homeostatic conditions.

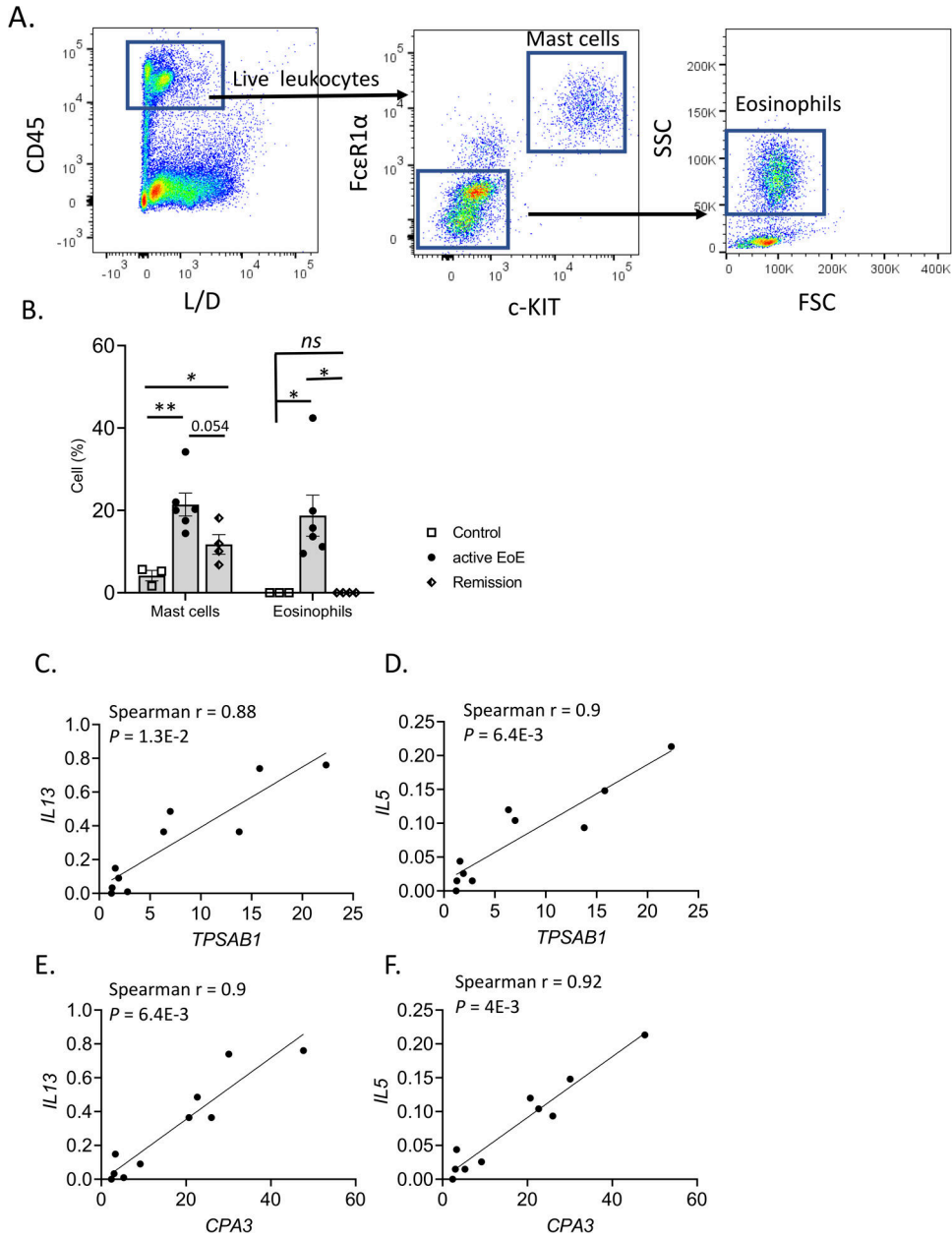


Figure 1: MC levels by EoE disease state and association with EoE cytokine milieu
A–B A cohort of esophageal tissue (control, n = 3; EoE, n = 6; EoE remission, n = 4) was processed to obtain a single-cell suspension. Cells were stained and analyzed via flow cytometry to identify mast cells (MCs) (CD45^{high}c-KIT^{high}FcεR1α^{high}); **(A)** the gating strategy is shown, and the L/D X-axis label represents staining for live/dead cells. **(B)** The percentage of mast cells and eosinophils out of the total white blood cell population (CD45^{high}) is graphed. Individual markers represent distinct individuals, and bars represent mean ± SEM, which was analyzed using a t-test for mean comparison between two groups; **, P < 0.01 * , P < 0.05; ns, not significant. **C–F** Spearman correlation of mast cell markers (*TPSAB1* and *CPA3*) with key gene expression levels (*IL13* and *IL5*) and comparison to other immunocyte markers in active EoE. Individual markers represent distinct individuals.

Data are derived from bulk RNA sequencing of patients with active EoE (n = 10), as reported^{6, 92}. P-value Bonferroni-Dunn's correction was calculated for each correlation.

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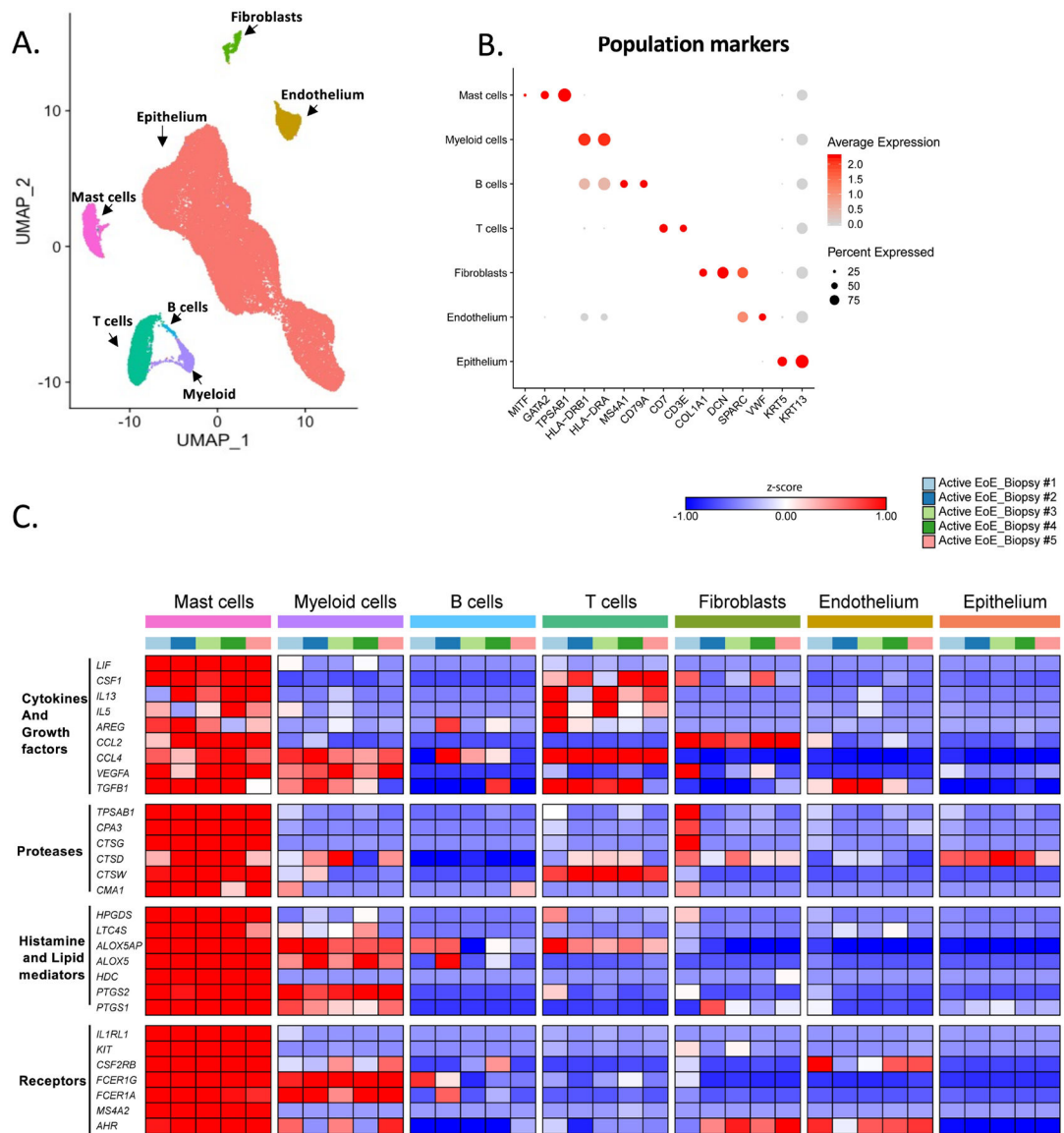


Figure 2: esophageal MC identification and characterization by scRNA-seq

A–B Examination of esophageal cell populations on the basis of gene expression. **(A)** Results were analyzed using an experimental workflow that led to a uniform manifold approximation and projection (UMAP) plot for dimension reduction displaying 39,562 single cells. The plot is colored by cell types derived from the shared nearest neighbor (SNN) clustering and enriched marker genes. Red, epithelium; yellow, endothelium; green, fibroblasts; magenta, mast cells (MCs); turquoise, T cells; light blue, B cells; and purple, myeloid cells. **(B)** A dot plot of marker genes for indicated cell types is shown. **C** Heatmap of MC-enriched genes for proteases, histamine and lipid mediator related, cytokines and growth factors and receptors in the 5 active EoE donors. The map's color for each gene is proportional to the average per-cell gene expression within the given patient in the esophageal cell populations. MCs were isolated from esophageal biopsies derived from patients with active EoE ($n = 5$) or remission EoE ($n = 3$) and non-EoE controls ($n = 2$).

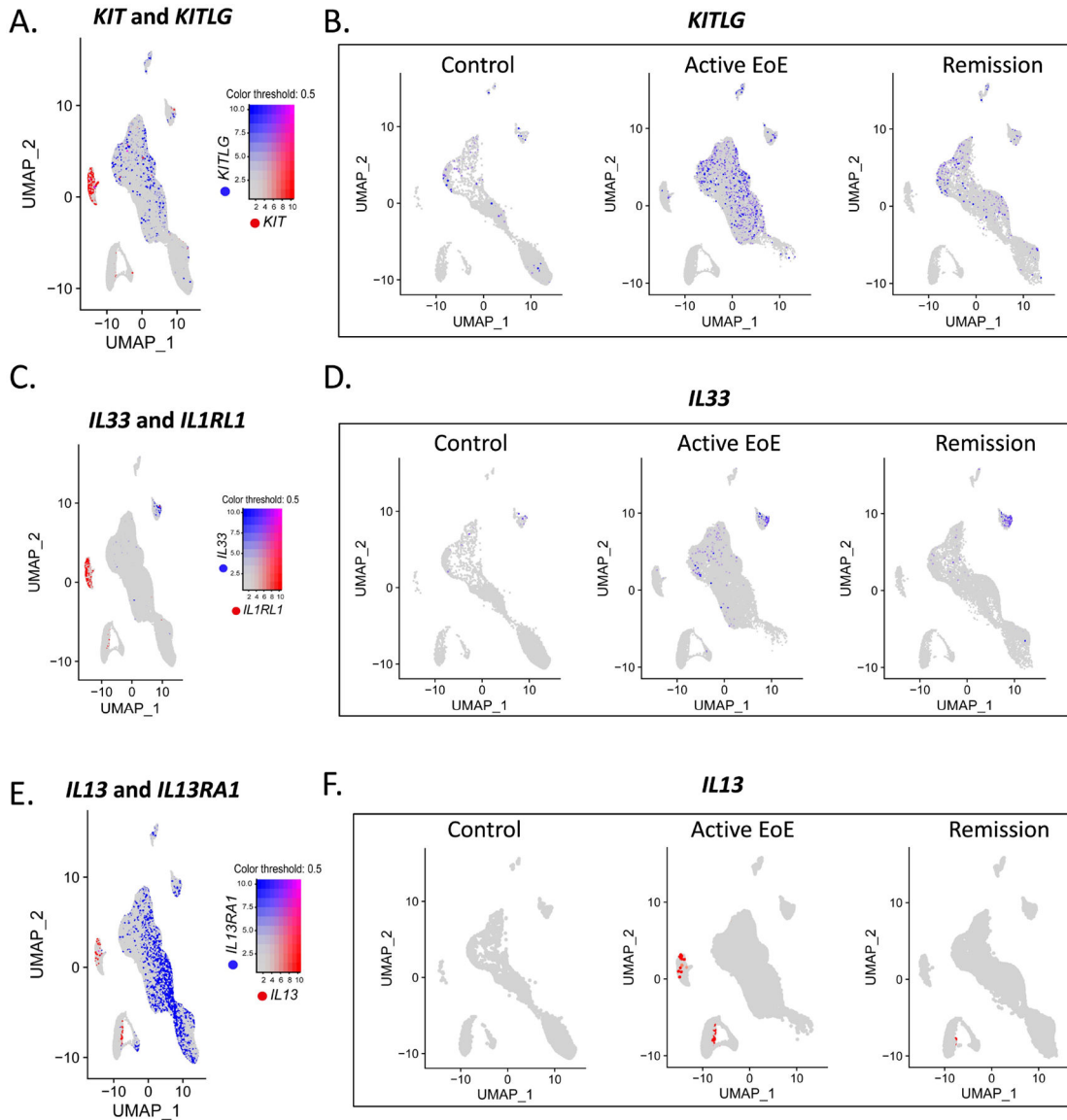


Figure 3: EoE-associated MCs axes

A–F Examination of mast cells (MC) potential interaction with other esophageal cell populations in cellular networking. Feature plot analyses of EoE hallmark genes of the scRNA-seq of the ten biopsies obtained from patients with active EoE (n = 5), remission EoE (n = 3), and non-EoE controls (n = 2). Feature plots of co-expression of the key type 2 cytokines and the compatible receptors in MCs (red) and other esophageal cell population(s) (blue) (A, C, E) and feature plots split by diagnosis (B, D, F) are presented.

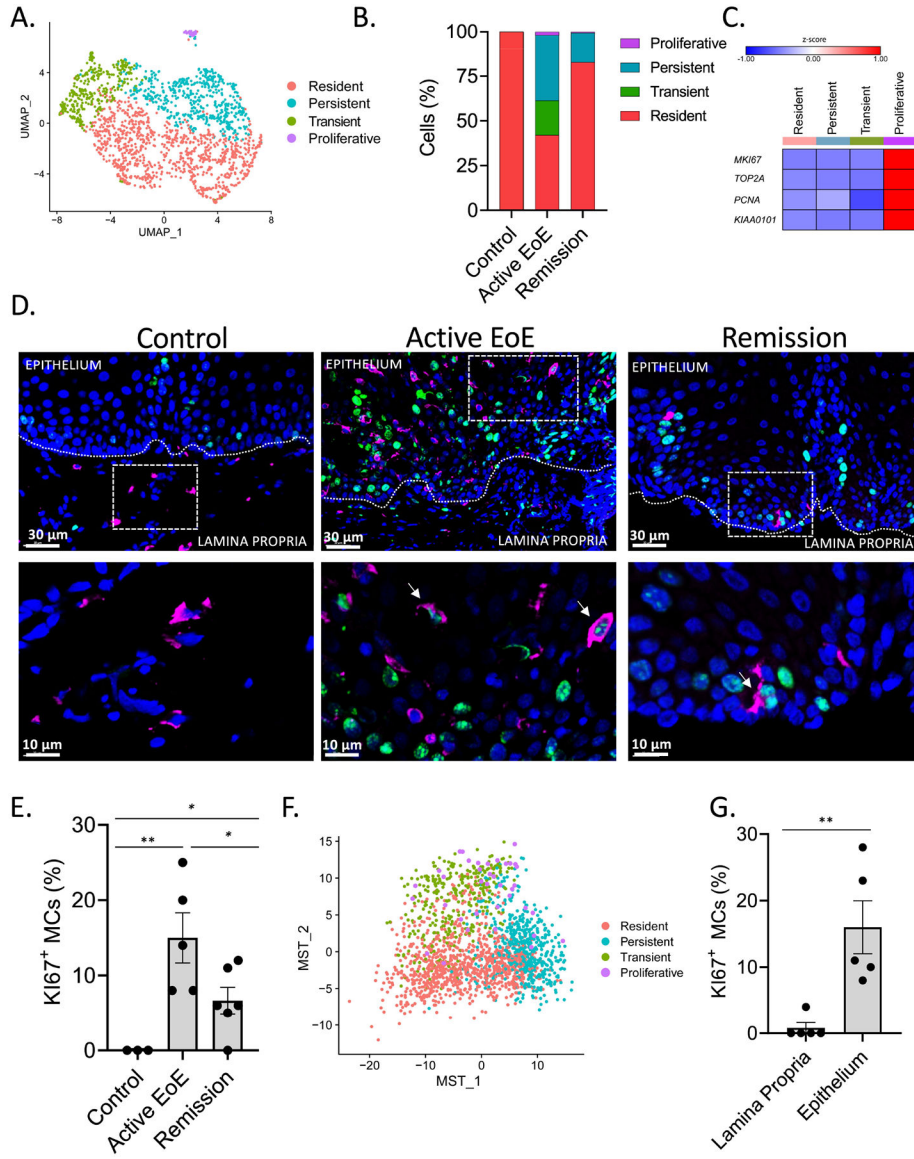


Figure 4: Esophageal MC populations across the diagnostic spectrum
A–C, F Examination of esophageal mast cell (MC) populations on the basis of gene expression. Esophageal biopsies were subjected to scRNA-seq. **(A)** Results were analyzed using an experimental workflow leading to a UMAP plot of 1,897 single mast cells (n = 10 samples) that were subsetted and reclustered from the total esophageal single cells (39,562 cells) colored by SNN clustering. Red, resident; green, transient; turquoise, persistent; and magenta, proliferative. **(B)** The stacked bar graph shows a given mast cell subpopulation as a percentage of the total mast cells in each sample. **(C)** Heatmap of proliferation marker genes; **(F)** MST plot of pseudotime analysis reclustered and organized mast cell populations on the basis of genetic similarity, colored by the original SNN clusters. **A, F** Individual markers represent individual cells. **D–E, G** A separate cohort of esophageal tissue (active EoE, n = 5; remission, n = 6; control, n = 3) was subject to immunofluorescent staining for tryptase (magenta), as a marker of MCs, and KI67 (green), as a marker of proliferation.

Representative pictures (**D**) and quantification of the percentage of KI67+ MC in the entire esophageal biopsy of all patients (**E**) and specifically in the epithelial and lamina propria in esophageal biopsies of patients with active EoE (**G**) are being shown in magnifications of 20X (**D**, upper row) and 100X (**D** lower row, magnified area of 20X indicated by dashed line boxes). **D**, double-positive cells for tryptase and KI67 depicted by the arrows **E**, **G** Individual markers represent distinct individuals, and bars represent mean \pm SEM, which was analyzed using the t-test for comparison between each two groups. *, $P < 0.05$, **, $P < 0.01$.

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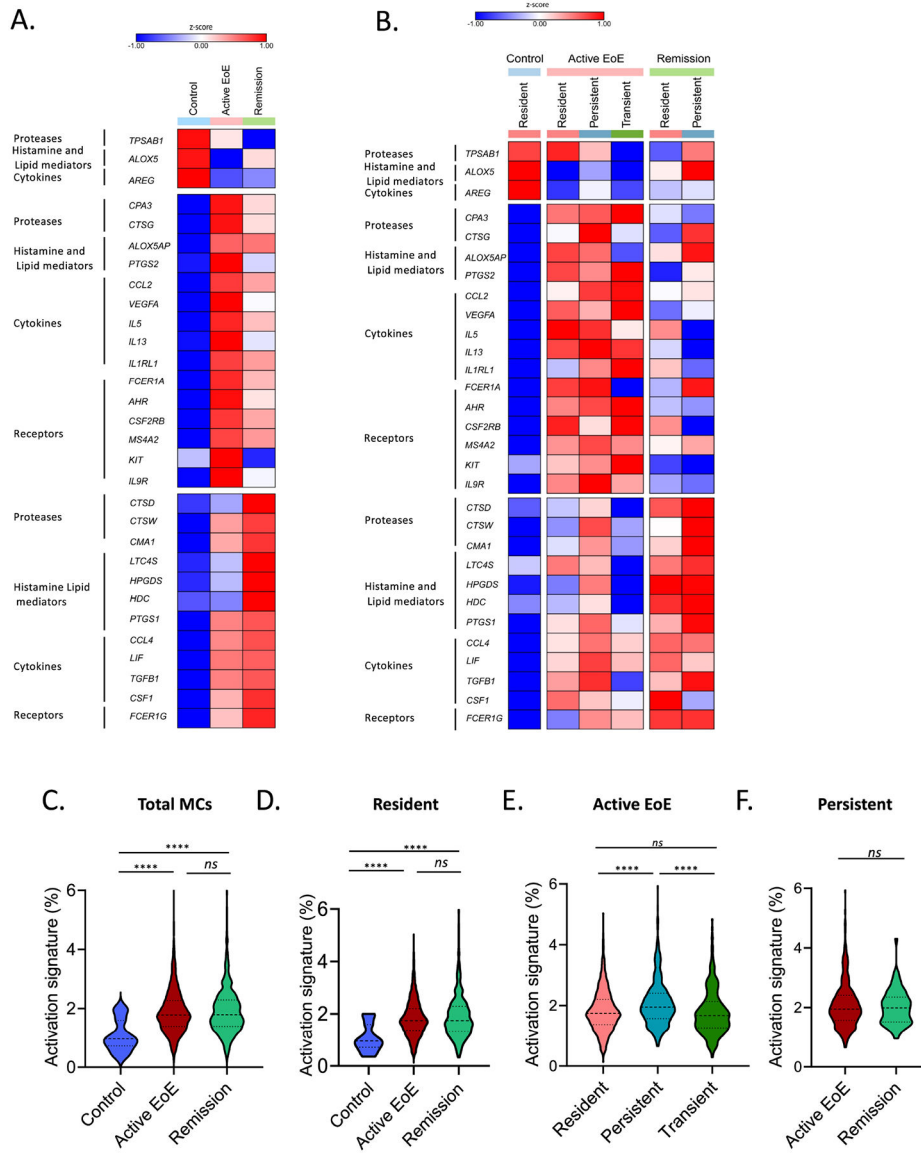


Figure 5: Characterization of the diversity of esophageal MC populations
A–F Examination of esophageal mast cell (MC) populations on the basis of gene expression. Heatmaps of enriched marker gene expression in the entire MC population (**A**) and divided by MC subpopulations (**B**) as a function of the disease state. The map’s color for each gene is proportional to the average per-cell gene expression within the given MC population. Violin plots indicate the percentage of genes out of the total gene number included in the activation signature expressed by the MC population as a whole (**C**), the resident population in each diagnostic state (**D**), all MC populations/cluster present in active EoE (**E**), and the persistent population in EoE compared to remission (**F**). Bars represent mean ± SEM, which were analyzed using one-way ANOVA with Tukey post-test; ns = not significant, ****, P 0.0001.

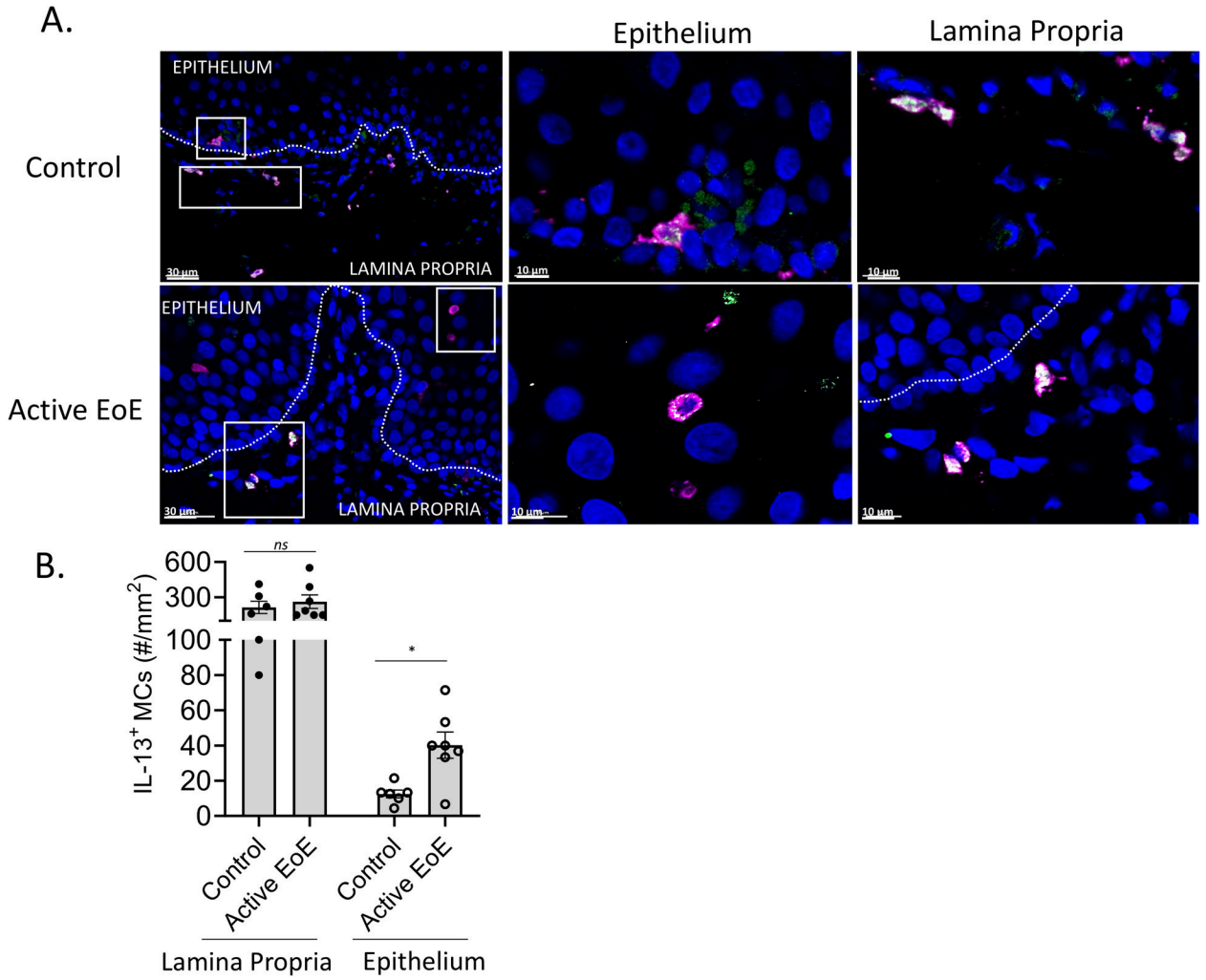


Figure 6: Examination of MC IL-13 protein expression

A–B A cohort of esophageal tissue (control, n = 6; active EoE, n = 7) was subject to immunofluorescent staining of tryptase (magenta) as a marker of mast cells (MCs) and IL-13 (green). **(A)** Representative pictures from control and active EoE are shown in magnification of 20X (left panels) and 40X (epithelium middle panel and lamina propria right panel). Dashed line boxes represent the magnified area in epithelium and lamina propria, respectively. Double positive staining display as a combination of magenta and green, generating white staining. **(B)** Quantification of IL-13⁺ MC numbers per mm² in the lamina propria and epithelial layers. **B** Bars represent mean ± SEM, which were analyzed using the t-test; *, P < 0.05, *ns* = not significant.