

Cell-by-cell deciphering of T cells in allergic inflammation



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Overall Purpose/Goal: To provide excellent reviews on key aspects of allergic disease to those who research, treat, or manage allergic disease.

Target Audience: Physicians and researchers within the field of allergic disease.

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Activity Objectives:

1. To understand the key T_H2 cytokines contributing to eosinophilic esophagitis (EoE).
2. To understand the characteristics of regulatory T (Treg)-like cells.
3. To understand which diseases show ineffective Treg-like cells accumulating in the gastrointestinal mucosa.
4. To become familiar with key findings in single-cell RNA sequencing (scRNA-seq) with allergic inflammation in the esophageal epithelium.

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Technical advances in single-cell RNA sequencing (scRNA-seq) render it possible to examine the transcriptomes of single cells in patients with allergic inflammation with high resolution in the context of their specific microenvironment, treatment, and disease status. Using a recently published scRNA-seq study of tissue T cells as an example, we introduce the major pipeline steps, illustrate the options of scRNA-seq platforms, summarize new knowledge gained from this study, and provide directions for future research. The presented scRNA-seq study elucidated the T-cell heterogeneity present in an allergic inflammatory tissue focused on eosinophilic esophagitis, a prototypic, chronic, allergic disease, which provided a unique opportunity to probe the pathogenesis of allergic inflammation at the tissue level through readily available endoscopically procured biopsy specimens. scRNA-seq analysis identified 8 populations of $CD3^+$ T cells and defined 2 disease-specific populations of $CD3^+CD4^+$

T cells, including a markedly activated type 2 cytokine-producing pathogenic cell population distinguished by expression of the short-chain fatty acid receptor free fatty acid receptor 3 and a population of regulatory T cells. In addition to presenting and interpreting new findings within the prior literature, we postulate about future single-cell next-generation sequencing platforms in this burgeoning field. (*J Allergy Clin Immunol* 2019;144:1143-8.)

Key words: T_H2 cells, eosinophilic esophagitis, scRNA-seq, T_H2 cytokine, food allergy

A typical human cell consists of a diploid genome composed of 2 copies of approximately 3 billion base pairs of DNA and more than hundreds of millions of bases of mRNA differentially

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Abbreviations used

EoE:	Eosinophilic esophagitis
FFAR3:	Free fatty acid receptor 3
NGS:	Next-generation sequencing
scCITE-seq:	Single-cell cellular indexing of transcriptomes and epitopes by sequencing
SCFA:	Short-chain fatty acid
scRNA-seq:	Single-cell RNA sequencing
TCR:	T-cell receptor
Treg:	Regulatory T

expressed by a myriad of cell types in the body. Advances in next-generation sequencing (NGS) have allowed profiling of the collection of mRNA species (the transcriptome) expressed in specific organs, tissues, and cells, but these advances have relied on analysis of bulk populations of cells, typically a mixture of millions of cells from isolated tissue or cell culture. With advances in single-cell capture and the automated cDNA library generation pipeline, it is now possible to examine the transcriptomes of single cells, a process referred to as single-cell RNA sequencing (scRNA-seq). This breakthrough technology allows unprecedented resolution of cellular differences and better comprehension of the function of individual cells in the context of their specific microenvironment, treatment, and/or disease contexts. Conceivably, the scRNA-seq platform can achieve many unique objectives beyond conventional methodology, including identification of rare cell populations, definition of disease subtypes, discovery of novel cellular markers, characterization of cellular heterogeneity and subsets, elucidation of disease mechanisms, and opportunities for precision and personalized medicine. The basic process of scRNA-seq involves isolation of single cells, nucleic acid extraction, RNA reverse transcription and amplification, cDNA library preparation (including NGS barcoding), NGS, and bioinformatic data analyses (typical pipeline is depicted in Fig 1).

To date, scRNA-seq has begun to be used in exploring circulating and tissue-residing cells in patients with select diseases, with a focus on cancer. Its usefulness has only recently been applied to studying allergic diseases. Eosinophilic esophagitis (EoE) provides a unique opportunity to probe the molecular and cellular mechanisms of human allergic inflammation because tissue is readily available from routine endoscopies, which can provide multiple research biopsy specimens from each patient. EoE is a prototypic severe allergic disorder mediated by gene-environment interactions¹ involving the interplay of the innate (mucosal epithelium and eosinophils) and adaptive (T cell) immune systems and driven by type 2 cytokines (T_H2 cytokines), especially IL-13. EoE provides an opportunity to scrutinize tissue-residing cells, including T cells, which comigrate into the esophageal mucosa with eosinophils. Driven by the goal to uncover mechanisms of type 2 immunity in tissue from patients with EoE, our team built a comprehensive platform by fluorescence-activated cell sorting the single-cell suspension isolated from enzyme-digested human esophageal biopsy specimens, eventually obtaining 1088 single CD3⁺ tissue T cells from the biopsy specimens of patients with active EoE, patients in disease remission, or those with no history of disease

(control subjects).² Guided by pilot experiments in mice, our team opted for the C1 Fluidigm system (Fluidigm, South San Francisco, Calif) as the platform for the scRNA-seq study^{3,4} because of its ability to directly and physically examine the single-cell chamber (thereby removing any chambers with >1 cell, suboptimal morphology, or both), quality control of the cDNA library before the NGS, lack of the 3' bias in synthesized cDNAs, and relatively deep numbers of NGS reads per cell.

In this study we aimed to answer the following questions: (1) What is the heterogeneity of tissue T cells? (2) How different are tissue CD3⁺ T cells from their circulating counterparts? (3) Do any EoE-specific T-cell subpopulations exist, and how do they causally contribute to pathogenesis? Although prior studies have identified increased levels of type 2 cytokines in allergic tissue,^{5,6} it remained unknown which cells produce these cytokines and the relative level of specific cytokine-producing cells, particularly in the tissue of patients with allergic inflammation. In the allergy field controversy exists about the cellular sources of cytokines, particularly those involved in EoE. Conventional wisdom focuses on CD4⁺ T cells⁷ and, more recently, type 2 innate lymphoid cells⁸ as the chief sources of T_H2 cytokines, but there is an emerging body of literature that supports T_H2 cytokine production by a variety of other cell populations, including CD8⁺ T cells,⁹ invariant natural killer T cells,¹⁰ specialized T_H2A cells,⁷ mast cells, and reprogrammed regulatory T (Treg) cells.¹¹ Accordingly, the scRNA-seq study determined the following key findings in the allergic inflammation seen in tissue from patients with EoE: (1) conventional CD3⁺ T cells produce large amounts of type 2 cytokines in the epithelial mucosa; (2) a relatively large fraction of tissue CD4⁺ T cells constitutively produce type 2 cytokines without the *ex vivo* T-cell stimulation that is typically required to detect T_H2 cytokine production *ex vivo* (eg, allergen, phorbol 12-myristate 13-acetate, and anti-CD3/CD28); and (3) although they are highly abundant and produce large amounts of type II interferons, CD8⁺ T cells do not substantially and directly contribute to type 2 cytokine production. As a caveat, all these findings are in the context of allergic inflammation in the esophageal epithelium.

The classical theory has proposed that the type 2 inflammation observed in tissue from patients with EoE originates as a result of local food allergen-stimulated T_H2 cells. Analysis of human T_H2 cells in the tissue is rather limited, with only 1 study showing augmented IL-5⁺ T_H cells in the blood of patients with active EoE.¹² For those with atopic diseases other than EoE, nearly all of the published studies focused on deciphering type 2 cytokines are based on tissue immunostaining or flow cytometric analysis. Furthermore, detection of T_H2 cytokine production in isolated cells has been confined primarily to cells that are polyclonally stimulated *in vitro*. In contrast, scRNA-seq analysis did not require *ex vivo* stimulation of T cells, perhaps (1) because we were probing the specific allergic inflammatory site and (2) because of the high sensitivity of the scRNA-seq technique. We found that type 2 cytokines were produced by approximately 10% of the freshly isolated/unmanipulated tissue T cells in tissue from patients with EoE, a phenomenon verified at the mRNA and protein levels. In the T_H compartment, T_H2 cytokine-capable T cells reached the surprisingly high percentage of greater than 30% CD4⁺ T cells in patients with EoE. This enrichment

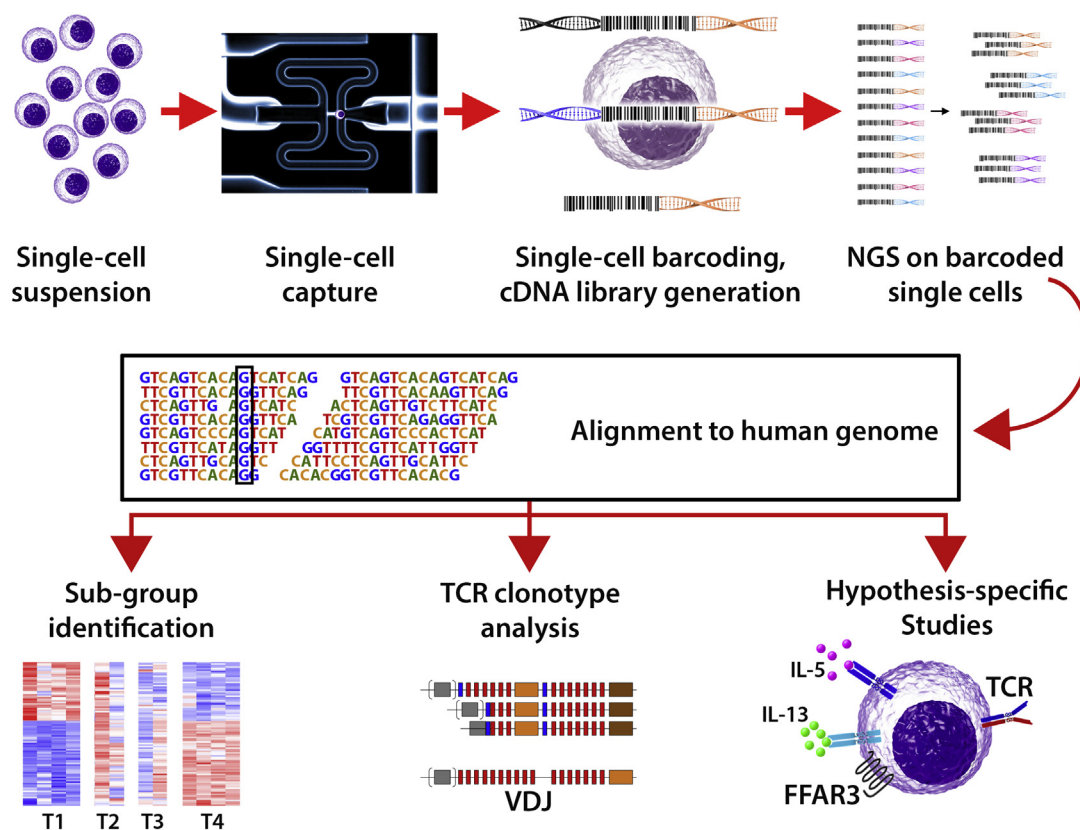


FIG 1. Schematic work flow of scRNA-seq with tissue cells from biopsy tissue. A visual summary of the major steps involved in the scRNA-seq platform that was applied to studying tissue cells isolated from biopsy specimens is shown. The major steps are listed sequentially, consisting of 4 indispensable modules: single-cell acquisition, single-cell barcoding (to ensure each single cell is specifically represented by a unique molecular DNA sequence), cDNA library generation, and NGS.

contrasts with the frequency of these cells in the blood, where they are typically less than 5% of CD4⁺ T cells, even in allergic patients.¹³⁻¹⁵ Enrichment of *bona fide* T_H2 cells in tissue from patients with EoE suggests that there is an active *in situ* differentiation process in addition to the recruitment of circulating T_H2 cells. Although scRNA-seq data indicated that the pathogenic effector T_H2 cells and Treg-like cells are largely CD103 (integrin α E, the reported intraepithelial lymphocyte marker) negative, whether these 2 populations of cells are mucosal-resident T cells or newly recruited T cells has not yet been determined.

Consistent with prior literature, the type 2 cytokine-producing effector T_H2 cells (eg, those producing IL-4, IL-5, and IL-13) also expressed mRNAs that encode for chemoattractant receptor-homologous molecule expressed on T_H2 lymphocytes (CRTH2, aka, prostaglandin D₂ receptor), ST2L (the IL-33 receptor), IL-17 receptor B (the IL-25 receptor), and hematopoietic prostaglandin D synthase (a rate-limiting enzyme involved in synthesizing prostaglandins in T_H2 cells).^{7,13} In addition, we identified expression of the mRNA for free fatty acid receptor 3 (FFAR3),² which encodes a short-chain fatty acid (SCFA) receptor.¹⁶ Focusing on the transcriptome specifically expressed in these tissue T_H2 cells, we used gene ontology analysis and identified a lipid-sensitive pathway that was centered on the FFAR3-SCFA axis and specific to allergic

tissue. Subsequently, we substantiated these findings by demonstrating that FFAR3 ligation (engaging butyrate and other SCFAs) enhanced type 2 cytokine production (*in vitro* and in a murine model *in vivo*) in mouse and human systems and, interestingly, that IL-4 induced FFAR3 expression during T_H2 cell development.

These data also suggested that butyrate, which can be derived from the microbiota¹⁷ and/or influenced by diet,¹⁸ might be a key positive regulator of type 2 immunity in the tissue from patients with EoE (Fig 2). Indeed, butyrate-producing bacteria are present in the human esophagus, and esophageal dysbiosis occurs in patients with EoE.^{19,20} These findings add controversy to the primary viewpoint that butyrate and other SCFAs have anti-inflammatory effects, perhaps mediated by alternative SCFA receptors on other cell types (eg, FFAR2 on Treg cells),²¹ in the lower gastrointestinal tissue compartment. Proposed interactions of the esophageal microbiota and butyrate-receiving pathogenic T_H2 cells are shown in Fig 2.

The C1 platform and the associated NGS read depth provided strong sequence coverage, allowing the collaborative team to examine for the presence of T-cell receptor (TCR) clonotype enrichment in patients with EoE. In contrast to the expected allergen-specific oligoclonality in tissue from patients with EoE, abundant TCR polyclonality in the EoE T-cell compartment in patients with active EoE was shown, as assessed by using analysis

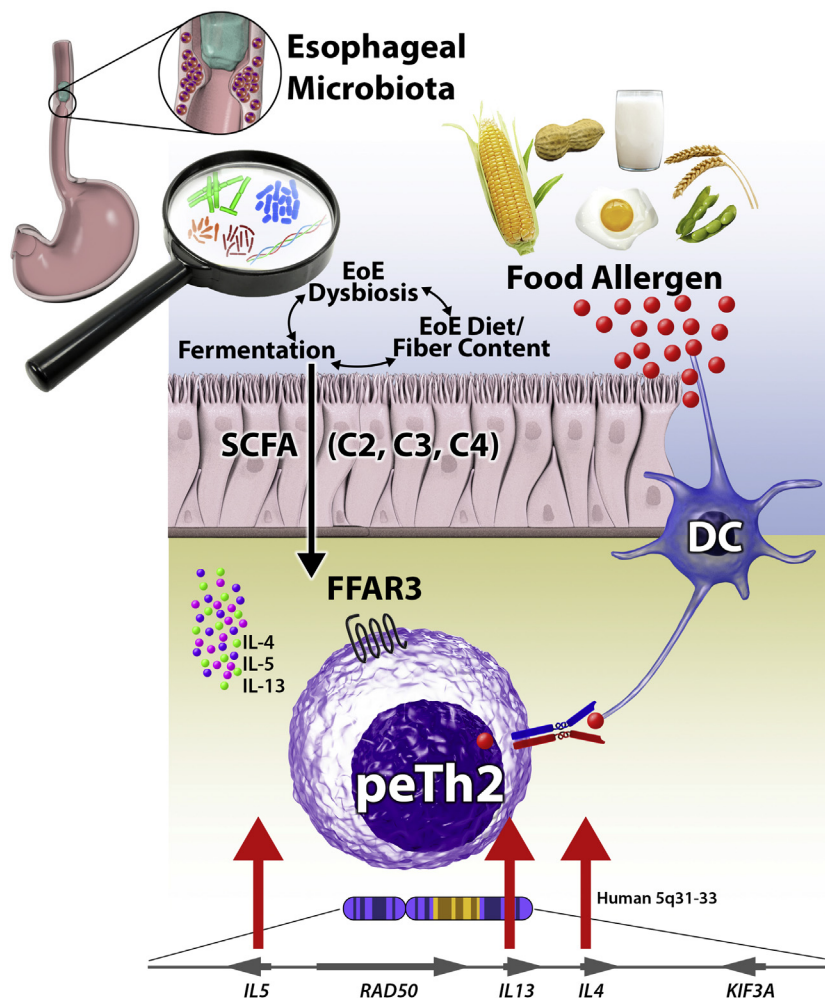


FIG 2. The proposed SCFA (butyrate)-mediated interactome of the microbiota, EoE diet, and effector T_H2 cells. The FFAR3-SCFA axis represents a novel and EoE-specific pathway identified by using scRNA-seq on tissue T cells. This finding could have a significant effect on upper gastrointestinal tract T_H2 mechanisms in patients with EoE because the mucosal immunocyte-luminal content interactome has been little known. The gastrointestinal luminal microbiota provides the source of butyrate as a major metabolite of the butyrate-capable flora. To a certain degree, the diet of a person with EoE would also contribute to variations in SCFA concentration because of dietary fiber differences. These 2 factors would also interact with the commensal flora dysbiosis observed in patients with EoE and collectively contribute to the T_H2 pathogenesis centered on tissue effector T_H2 cells. DC, Dendritic cells; *peTh2*, pathogenic effector T_H2 cells.

of Shannon entropy. Likewise, patients with active disease had the most diversified TCR clonality. One potential interpretation of the observed EoE TCR polyclonality is 2 fold: (1) 500 T cells are not a robust number for statistical power in this type of analysis and/or (2) even though the TCR sequences can be reliably extracted, current bioinformatic tools might not readily allow precise prediction of allergen reactivity.²² Allergen-reactive TCRs are highly diversified and dynamic,²³ meaning that multiple TCR clonotypes could react to the same food allergen across a myriad of epitopes that evade the oligoclonality screening. Recently, a new chemistry system by 10x Genomics (Pleasanton, Calif) was developed for TCR compatibility,²⁴ providing larger scale and depth for TCR analyses.

scRNA-seq of the esophageal mucosa identified 2 disease-associated T-cell subpopulations: a population of Treg

cell-like $CD4^+$ T cells (enriched in patients with EoE) and a population of pathogenic effector T_H2 cells (specific to patients with EoE). Intriguingly, even though Treg-like cell counts also increase in patients with EoE and outnumber pathogenic T_H2 cells, influx of Treg-like cells does not successfully suppress allergic inflammation in patients with active disease. Our findings demonstrate that these 2 cell types share some gene expression profiles, such as *GATA3* and *CD25/IL2RA* (but not type 2 cytokine production), and do not yet substantiate that these 2 populations can transition between each other in tissue.^{25,26} The lack of optimal Treg cell inhibition could be due to (1) insufficient cell numbers that are overwhelmed by the *bona fide* T_H2 cells, (2) inhibitory signal from the microenvironment and antigen-presenting cells, (3) temporal and spatial issues that are not addressed by single-timepoint studies, (4) lack of

costimulation signals and induction agents (eg, retinoic acid and hyaluronic acid),²⁶⁻²⁸ and/or (5) lack of sufficient TGF- β expression or signaling essential for induced Treg cell function in the peripheral gastrointestinal mucosal tissue.^{29,30} Notably, EoE is not the only disease with ineffective Treg-like cells accumulating in the gastrointestinal mucosa because this paradox is also observed in patients with inflammatory bowel disease.^{31,32}

Future studies are expected to elucidate the *bona fide* suppressive capacity of esophageal Treg cells and ways to enhance their cellular and molecular effectiveness. The identified tissue T_H2 cells in the esophagus resemble the recently published pathogenic effector T_H2 cells¹³ and allergy-inducing T_H2A cells.⁷ However, translating observations from patients with esophageal allergic inflammation might not apply equally to allergic inflammation in other tissues.

Biopsy tissue from patients with EoE has proved to be an invaluable tool to understand the cellular immunology of this allergic disorder because a plethora of leukocytes and other relevant cells (eg, epithelial and endothelial cells) are readily retrievable from inflamed tissue.³³ The C1 Fluidigm platform is limited by the number of cells analyzed and its incapacity to use a barcoded antibody for labeling the surface protein of interest (as can be done with single-cell cellular indexing of transcriptomes and epitopes by sequencing [scCITE-seq]).³⁴ Going forward, an emerging new scRNA-seq platform (10x Genomics) provides an opportunity for greater throughput means to expand scRNA-seq to by at least 10-fold greater cell numbers; this will likely uncover an even more precise understanding of the distinct functions of each cell type and the interactive cellular mechanisms in patients with allergic inflammation. For example, even though EoE is named and centered around eosinophil-associated inflammation, we know relatively little about the molecular and cellular function of eosinophils directly in patients with this disease, a subject with direct therapeutic implications given the recently emerging new classes of biological agents that now target eosinophils.³⁵ scRNA-seq of infiltrating eosinophils in tissue from patients with EoE will likely be a key step in understanding this cell type, its probable heterogeneity, and its role in patients with EoE.

scRNA-seq analysis is part of the dawn of a new era of fine-resolution technologies that are revolutionizing translational research. The field of single-cell sequencing is rapidly evolving; for example, single-cell analyses that focus on epigenomics (eg, DNA methylome sequencing and single-cell assay for transposase-accessible chromatin with sequencing [scATAC-seq])³⁶ and protein expression (eg, scCITE Seq)³⁴ are now becoming readily available. From an anatomic perspective, spatial transcriptomics³⁷ and paired-cell scRNA-seq³⁸ provide opportunities to elucidate diseased processes cell by cell in the context of *in vivo* tissue structure. This might aid in understanding the interaction of disease-specific Treg and T_H2 cells in patients with EoE. Capitalizing on these advances will be key in pursuing a better understanding of molecular causes, identifying novel pharmaceutical targets, and eventually treating allergic diseases by modulating the immune system.

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What is known?

- There is clinically relevant heterogeneity in tissue T cells.
- Despite CD8⁺ T-cell dominance in esophageal tissue, T_H2 cytokines are produced by CD4⁺ T cells.
- There is an EoE-specific “superactivation” of the CD4⁺ tissue T-cell compartment.
- CD4⁺ T cells in allergic inflamed tissue are notably enriched (approximately 30%) in T_H2 cytokine production compared with CD4⁺ T cells in the circulation (typically <5%).
- An unexpectedly high prevalence of Treg-like cells coexist in allergic tissue, which likely contributes to the chronicity and phenotype of the disease.
- Key surface markers and transcription factors expressed by tissue T_H2 cells are notably enriched in an active lipid sensory pathway that includes the receptor for SCFAs (FFAR3).
- Butyrate and other SCFAs might contribute to disease pathogenesis. The data presented argue for a positive contributory role.

What is still unknown?

- Is there an allergen-specific TCR repertoire beyond oligoclonality, prompting large-scale, high-throughput TCR clonotyping?
- Can the plasticity of Treg cells, if they exist in tissue from patients with EoE *in situ*, be modulated for the treatment of EoE?
- What is the relative contribution of T_H2 cytokines from non-T cells, such as type 2 innate lymphoid cells and mast cells, in the epithelium and lamina propria?
- Will scRNA-seq improve the understanding and eventual effectiveness of EoE treatments?
- Do the observations and conclusions from this scRNA-seq study apply to other tissues with allergic inflammation?

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