*Review article*

# Revealing the heterogeneity of CD4$^+$ T cells through single-cell transcriptomics

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**Single-cell RNA sequencing (scRNA-seq) offers the ability to resolve whole transcriptomes of single cells with substantial throughput, and it has revolutionized studies of gene expression. The transcriptional resolution available can uncover fine structures of biologic heterogeneity that are manifest among cell populations. Here, we review the applications of scRNA-seq to profile the phenotypes and clonotypes of CD4$^+$ T cells. First, we describe challenges inherent to scRNA-seq that are important for analysis of CD4$^+$ T cells, as well as the technical solutions that are emerging to address these challenges. We then consider major themes of the application of scRNA-seq to CD4$^+$ T cells, including investigation of CD4$^+$ T-cell heterogeneity in model systems, analysis of populations from the peripheral blood, and the profiling of tissue-resident populations. We place emphasis on capabilities unique to scRNA-seq, such as the ability to obtain paired T-cell receptor and transcriptome information from single T cells and the potential to elucidate interactions between CD4$^+$ T cells and other cells in their environment. Finally, we conclude by considering future areas of technologic advancement and innovation through which scRNA-seq may further shape our understanding of the roles of CD4$^+$ T cells in health and disease. (J Allergy Clin Immunol 2022;150:748-55.)**

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CD4$^+$ T cells play a central role in coordinating adaptive immune responses.[1,2] Individual naive CD4$^+$ T cells bear a specificity for antigen–MHC class II complexes that results from expression of a single, uniquely rearranged T-cell receptor (TCR). These cells are initially activated following stimulation via interactions with a cognate antigen–MHC class II complex. Subsequently, they undergo clonal expansion and differentiation into one of a variety of subtypes, including helper T cells ($T_H1$, $T_H2$, $T_H17$, and follicular helper T [$T_{FH}$] cells) and regulatory T (Treg) cells.[1-11] This process of differentiation is guided by signals available in the extracellular milieu, and the resulting lineages of CD4$^+$ T-cell subtypes are defined predominantly by select transcription factors (ie, T-bet, Gata3, Ror-γt, Bcl6, and Foxp3).[1,2,4,12] The functional traits of each CD4$^+$ T-cell subtype include distinct cytokine profiles that yield distinct effector functions after a future encounter with antigens. A subset of expanded CD4$^+$ clonotypes can also undergo differentiation into memory CD4$^+$ cells, leading to the establishment of immunologic memory.[13,14] In general, analytical characterizations of CD4$^+$ T cells aim to resolve the phenotypes and specificities of these cells present within a particular sample and uncover the biologic relationships within, such as those that may contribute to health or disease.

Single-cell RNA sequencing (scRNA-seq) currently affords the ability to analyze single cells with whole-transcriptome resolution, offering higher dimensionality than previous approaches for analyzing individual cells, such as flow or mass cytometry, which often rely on investigator-curated panels of markers, albeit with a modest reduction in throughput (thousands to tens of thousands of cells).[15-17] Consequently, scRNA-seq is well suited to the study of CD4$^+$ T cells, which comprise a functionally heterogenous population that also exhibits a level of plasticity.[12,18,19] In addition, scRNA-seq is compatible with a diverse array of sample types, including samples from the peripheral blood and tissue biopsy samples obtained from human patients. These features have made scRNA-seq an increasingly important analytic technology in both immunology and the broader biological sciences in the past 10 years.

In this review, we describe advances in our understanding of CD4$^+$ T-cell immunology enabled by scRNA-seq. We focus first on unique challenges encountered in the application of scRNA-seq to CD4$^+$ T cells and identify technical solutions that are

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*Abbreviations used*
EoE: Eosinophilic esophagitis
HDM: House dust mite
Treg: Regulatory T
scRNA-seq: Single-cell RNA sequencing
TCR: T-cell receptor

emerging to address these challenges. We then describe 3 key applications of scRNA-seq to the study of $CD4^+$ T cells, including profiling of $CD4^+$ T-cell responses in model systems, analyzing $CD4^+$ T-cell responses in samples obtained from peripheral blood, and understanding key functionalities of tissue-resident $CD4^+$ T-cell populations. Lastly, we conclude with a discussion of developing and future capabilities of scRNA-seq and consider how these advancements are poised to further extend our knowledge of $CD4^+$ T-cell immunology.

## ANALYSIS OF $CD4^+$ T CELLS WITH scRNA-seq

$CD4^+$ T cells are traditionally classified into discrete subtypes, including $T_H1$, $T_H2$, $T_H17$, and $T_{FH}$ cells, which are defined by the expression of distinct transcription factors regulating their differentiation and the expression of cytokines and other effector genes that mediate their functions.[6-11] These key transcripts, however, are not necessarily among the most highly expressed genes, and they constitute only a small fraction of the total mRNA present in a single T cell. Thus, to accurately annotate $CD4^+$ T-cell phenotypes present in scRNA-seq data, the chemistries used for amplification and library preparation must be sufficiently sensitive to ensure robust recovery of these defining transcripts. "Gene dropout" (ie, the spotty detection of gene expression in single-cell libraries that stems from incomplete recovery of cellular mRNA) is exceedingly common in single-cell data analysis.[20,21] To address this challenge, recent advances in the molecular biology and chemistries for preparing libraries of cDNA for sequencing have included approaches based on the synthesis of a randomly primed second-strand of cDNA as an alternative to a 5-template switching reaction.[22] Such improved conversion of the mRNA yields libraries with enhanced informational complexity, offering improved scalability and increased sensitivity to detect these key transcripts. Improvements in the efficiencies of transcript capture and amplification for sequencing directly enhance the ability of scRNA-seq to resolve phenotypes of $CD4^+$ T cells.

Second, to analyze clonal relationships between T cells and to properly place individual T cells in the context of a response against a particular antigen, it is useful to recover both phenotypic information based on the transcriptome of a T-cell and knowledge of the T cell's rearranged TCR. Early demonstrations of single-cell sequencing based on the isolation of individual cells into microliter plates enabled the reconstruction of TCR rearrangements *in silico*.[23,24] By their nature, these solutions exhibit reduced throughput compared with massively parallel platforms for scRNA-seq, which utilize droplet encapsulation or microwell isolation.[25-28] In contrast, because massively parallel platforms emphasize short gene reads to obtain digital counts of gene expression instead of performing full-length RNA-seq, the depth of coverage for the TCR variable regions obtained with these platforms is very poor, limiting their ability to accurately identify specific TCR rearrangements from most T cells. Recently, new strategies compatible with massively parallel library constructions have developed for recovery of paired TCR-α/β variable region sequences, including strategies utilizing specialized RNA capture reagents,[29] methods for targeted sequencing of TCR-enriched libraries,[30,31] and commercially available T-cell–specific kits, such as the 10*x* Genomics 5' V(D)J + 5' Gene Expression kit (10× Genomics, Pleasanton, Calif). As a result, scRNA-seq has become one of the most effective methods to obtain paired TCR-α/β sequences that are matched with transcriptional profiles of the same cells.

Lastly, T cells specific for any individual antigen are rare in easily accessible samples, such as blood from a human patient.[32-34] In tissue or tumor biopsy samples, antigen-specific T cells may be expected to be enriched, but these samples are more difficult to obtain and the total number of cells available from these samples is often limited. These factors place an upper bound on the number of $CD4^+$ T cells that can be reasonably obtained in many contexts. Accordingly, robust studies of $CD4^+$ T cells require platforms for scRNA-seq that are compatible with sparse cellular input while maintaining high rates of cell and gene recovery and throughput sufficient to detect transcriptional structures through unsupervised analysis. Platforms based on the physical isolation of cells into subnanoliter wells, such as Seq-Well, BD Rhapsody (BD Biosciences, Franklin Lakes, NJ), or the Honeycomb Hive (Honeycomb Biotechnologies, Waltham, Mass), rather than the encapsulation of single cells into reverse-emulsion droplets, have demonstrated efficient rates of cell recovery as well as compatibility with the technical advancements already described.[25] In addition, gentle gravity-based loading of cells imposes less stress on cells than droplet encapsulation, better preserving cell viability and *ex vivo* T-cell phenotypes.[35]

## REDEFINING T-CELL IDENTITIES IN MODEL SYSTEMS

scRNA-seq enables analyses of whole transcriptomes from individual cells with minimal bias rather than relying on curated sets of markers for analysis. As a result, scRNA-seq has enabled re-evaluation of classical $CD4^+$ T-cell phenotypes defined on the basis of surface expression of a small set of protein markers. Comparison of the transcriptomes of single $CD4^+$ T cells with their surface phenotype has suggested that whereas some classic T-cell subsets, such as naive $CD4^+$ T cells and memory $CD4^+$ T cells are dominated by a single, representative transcriptional phenotype, other classic T-cell subsets, such as $CD4^+$ $T_H$ cells and $CD4^+$ Treg cells, comprise multiple distinct transcriptional phenotypes, suggesting that these higher resolution transcriptional phenotypes can be more representative of the key features of a T-cell population.[36,37]

scRNA-seq has also enabled the discovery of rare, discrete populations of T cells with previously undescribed functionalities (Fig 1, *A*). For example, a subpopulation of IL-13–producing $T_{FH}$ cells required for the production of high-affinity IgE was discovered by analyzing sorted PD-$1^+$CXCR$5^+$CD$44^+$ $CD4^+$ $T_{FH}$ cells in a model of allergic sensitization.[38] Profiling of IL-$10^+$ $CD4^+$ T cells with scRNA-seq in a mouse model of inflammatory bowel disease identified a proinflammatory population of IL-$10^+$Foxp$3^-$ $CD4^+$ cells.[39] Similarly, analysis of IL-$10^+$ $CD4^+$ T cells

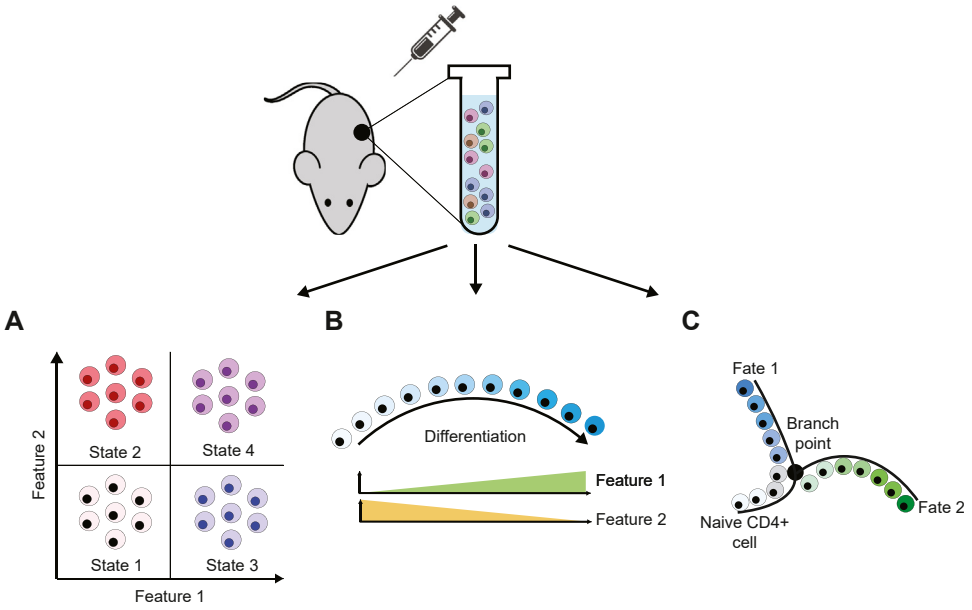


**FIG 1.** Conceptualization of CD4$^+$ T cell identities with scRNA-seq. **A**, Classification of T-cell phenotypes into discrete phenotypes defined based on binary expression of select features (ie, transcription factors, cytokines). **B**, Conceptualization of T-cell phenotypes as related by a continuum of differentiation. Select features may vary in expression level as a function of location on this continuous trajectory. **C**, Analysis of fate decisions among CD4$^+$ T cells. Naive T cells gradually differentiate into distinct heterogeneous fates as they undergo activation and expansion. Branch points on this trajectory represent states in which commitment is made of one of these heterogeneous fates.

recovered from the spleens of mice responding to chronic infection with lymphocytic choriomeningitis virus demonstrated the presence of IL-10$^+$IL-21$^+$ $T_{FH}$ cells necessary for sustaining germinal center reactions and humoral immunity during chronic infection.[40] A second study of lymphocytic choriomeningitis virus profiled CD44$^{high}$ and GP66-specific CD4$^+$ T cells and identified a small cluster of T cells with a central memory precursor phenotype.[41] This study demonstrated that an upregulated transcription factor, *Thpok*, prevented the emergence of an effector-like transcriptional program in this precursor population and promoted memory differentiation. These studies have used scRNA-seq to extend our knowledge of discrete CD4$^+$ T-cell phenotypes by uncovering new T-cell subtypes and functionalities associated with novel combinations of cytokines and transcription factors.

Another feature of scRNA-seq analysis yielding novel insights when applied to CD4$^+$ T cells is the conceptualization of phenotypic states as related by a continuum of differentiation, rather than as completely discrete populations (Fig 1, *B*). These analyses often aim to construct cellular trajectories by utilizing bioinformatic tools to construct pseudotemporal orderings or analyze RNA velocity.[42-46] For example, an analysis of CD4$^+$ T cells either undergoing $T_H17$ cell differentiation *in vitro* or obtained from the central nervous system of mice with experimental autoimmune encephalitis established a phenotypic continuum present among these cells and identified novel regulators associated with a pathogenic axis of $T_H17$ differentiation.[47] An "effectorness gradient" spanning from naive to central to effector memory T cells and acting as a determinant of cytokine responses has been established among CD4$^+$ T cells undergoing *in vitro* polarization with different combinations of cytokines.[48] Rather than occupying discrete phenotypic states, colonic T cells have been suggested to lie within a polarized effector continuum that exhibits skewing in response to microbial or infectious perturbation.[49] These studies present alternative, continuous models of CD4$^+$ T-cell identities, in contrast to traditional models that classify CD4$^+$ cells into discrete phenotypes.

scRNA-seq is also particularly well suited to studies of CD4$^+$ T-cell differentiation and plasticity (Fig 1, *C*). An approach based on temporal mixtures of gaussian processes defined trajectories of $T_H1$ and $T_{FH}$ cell lineages in a model of *Plasmodium* infection and revealed that single T-cell clones bifurcate and populate both fates in this context.[50] Longitudinal measurements with scRNA-seq in a mouse model of graft-versus-host disease revealed the divergence of alloreactive CD4$^+$ T cells into either an effector fate, which exhibited cytokine expression, or a quiescent state, which exhibited minimal cytokine production but maintained recall potential following secondary transplantation.[51] scRNA-seq measurements in a "provenance mapping" mouse model that uses a photoconvertible protein to track the location of T-cell priming in experimental autoimmune encephalitis demonstrated distinct phenotypic profiles and homing patterns between T cells primed in either the mediastinal or inguinal lymph node.[52] Overall, these studies provide new methodologies for the analysis of CD4$^+$ T cells and have developed new insight into the mechanisms through which single clones of CD4$^+$ T cells differentiate into heterogeneous fates.

## PROFILING OF PERIPHERAL CD4$^+$ T-CELL RESPONSES *EX VIVO*

The profiling of antigen-specific T-cell responses from peripheral blood is often used as a tool to understand the magnitude and

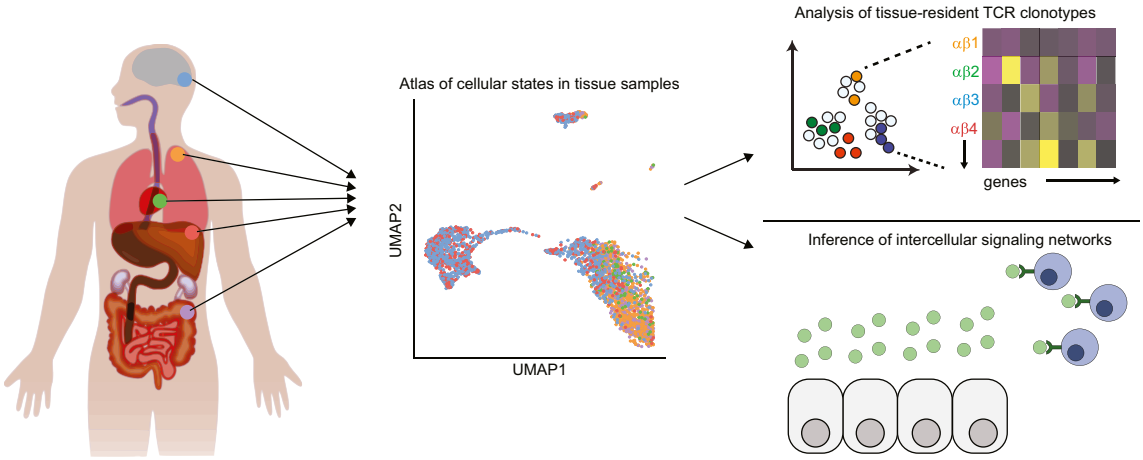


**FIG 2.** Analysis of tissue-resident CD4$^+$ T cells with scRNA-seq. Cells recovered from biopsy samples from various tissues can be analyzed with scRNA-seq, allowing the construction of high-resolution analysis of tissue-resident CD4$^+$ T-cell populations and other cell phenotypes in their microenvironment. These atlases enable the elucidation of T-cell phenotypes present in biopsy samples and can support the analysis of tissue-resident TCR clonotypes. They also allow the inference of intercellular signaling networks in these tissues, which can establish the mechanisms through which CD4$^+$ T cells interact with other cells present in their tissue niche.

quality of CD4$^+$ T-cell responses against a particular antigen. Although blood samples are easily obtained from human patients, the abundance of cells specific for a particular antigen of interest can vary widely depending on the disease context.[32-34] Thus, when analyzing CD4$^+$ T cells from the peripheral blood with scRNA-seq, it is important to consider exactly how to isolate a sufficiently large and enriched target population of T cells.

In some samples, such as peripheral blood samples obtained during acute viral infection or following vaccination, the magnitudes of antigen-specific T-cell responses present in the peripheral blood may be sufficient to allow reliable analysis of these cell populations without prior enrichment. In combination with a novel strategy for gene module analysis and cell-cell signaling network analysis, scRNA-seq analysis of PBMCs from samples collected during the acute phase of HIV infection revealed that peripheral CD4$^+$ T cells uniquely upregulated genes downstream of proinflammatory cytokines.[53] Analyses of peripheral blood samples from patients with COVID-19 using scRNA-seq have highlighted clonal expansions of CD4$^+$ T cells expressing cytotoxic signatures and have demonstrated the reactivities of these populations with COVID-19–derived antigens.[54-56] scRNA-seq has also revealed distinct *FOXP3*$^{high}$ and *MKI67*$^{high}$ differentiation paths among Treg cells present in the peripheral blood that were conserved in patients receiving allogenic hematopoietic stem cell transplantation.[57]

Class II tetramer reagents have previously been used to isolate antigen-specific CD4$^+$ T cells for scRNA-seq,[58,59] but 2 limitations of these reagents are their dependence on previously identified antigens and HLA types and the challenges in synthesizing reagents compatible with a diverse array of antigens and HLA types. As an alternative, many scRNA-seq studies have isolated antigen-specific CD4$^+$ T cells using functional behaviors, such as the upregulation of activation markers (often CD154, CD137, and/or CD69), following culture with antigen *ex vivo*.[60-62] This approach exhibits minimal bias for specific epitopes or HLA types, but it may also enrich a fraction of non–antigen-specific T cells activated indirectly though cytokine signaling pathways (often referred to as "bystander activation").[63] Studies of allergen-reactive CD4$^+$ T cells in food allergy have repeatedly demonstrated heterogeneity among allergen-reactive cells, including the detection of highly polarized $T_H2A$ cells in the peripheral blood.[64-68] A signature comprising an interferon response has been identified among house dust mite (HDM) allergen–reactive CD4$^+$ T cells isolated from the peripheral blood, and expression levels of this signature among $T_H$ and Treg cells differentiated asthmatic patients with HDM allergy from asthmatic patients without HDM allergy.[67] In addition, paired TCR sequences obtained from single-cell sequencing of antigen-reactive CD4$^+$ T cells have been used to identity the peptide epitopes recognized these T cells in patients with type I diabetes and autoimmune hepatitis.[69,70]

Longitudinal studies of peripheral blood samples with scRNA-seq have also enabled the tracking of select clonal lineages in human patients over time. Hu et al isolated peripheral CD4$^+$ T cells by using class II tetramer reagents from longitudinal samples obtained from patients with melanoma who were receiving personalized neoantigen vaccines.[59] scRNA-seq analysis of these cells revealed neoantigen-specific clonotypes that could be detected at multiple time points and that after patients had received neoantigen vaccines, neoantigen-specific cells transitioned into differentiation state from an initial naive state, followed by states characterized by signatures associated with effector function and activation-induced cell death, and finally to a memory state. In addition, scRNA-seq paired with TCR sequencing of peanut-reactive CD4$^+$ T cells from 12 patients with peanut allergy revealed a diversity of clonally restricted phenotypic states present among peanut-reactive CD4$^+$ T cells.[66] A longitudinal analysis of the clonotypes recovered in this study demonstrated that outcomes of peanut oral immunotherapy were associated with reprogramming rather than with deletion of $T_H2A$ cell–like clonotypes. Similar studies could provide insight into the mechanisms through which immunotherapies activate and reprogram clonotypic lineages of T cells and could also enable noninvasive monitoring of antigen-specific CD4$^+$ T-cell lineages in the peripheral blood.

## UNDERSTANDING TISSUE-RESIDENT CD4$^+$ T-CELL POPULATIONS

Many previous studies have used scRNA-seq to survey the cell populations within biopsy samples, allowing construction of cellular atlases of tissues or diseases of interest[71-73] (Fig 2). Such atlases have generated an improved understanding of tissue-resident CD4$^+$ T-cell phenotypes across a wide spectrum of diseases. For example, $T_H2$ cells that exhibit a simultaneous upregulation of $T_H2$ cytokines, receptors for epithelial-derived cytokines, and genes associated with prostaglandin synthesis have been identified in tissue samples across a wide range of allergic diseases, including eosinophilic esophagitis (EoE), atopic dermatitis, chronic rhinosinusitis, and asthma.[35,74-79] Studies of cancer biopsy samples have also identified novel CD4$^+$ T-cell phenotypes,[80-82] such as cytotoxic CD4$^+$ T cells, which have been demonstrated to display cytotoxic activity against autologous tumor cells.[81,82]

Tissue samples also provide a unique context for scRNA-seq studies to leverage TCR data. Many studies have identified clonotypic T-cell expansions associated with select CD4$^+$ T-cell phenotypes, highlighting the potential relevance of certain populations in the disease contexts under investigation.[35,66,78,80] In addition, the integration of TCR sequences with single-cell transcriptome data can be used to identify potential relationships between T-cell phenotypes. For example, a study of colon cancer demonstrated TCR sharing between tumor-resident Treg cell and other $T_H$ cell phenotypes, suggesting that these are induced Treg cells generated from the polarization of preexisting $T_H$ cell phenotypes.[83]

When samples from multiple tissue sites are available, the TCR can also be used as a unique barcode to track T-cell lineages across different locations. scRNA-seq profiling of CD4$^+$ T cells from different regions of the human colon and revealed that whereas CD4$^+$ T-cell clonotypes were shared between the proximal and sigmoid colon, the sigmoid colon was enriched for clonally expanded $T_H1$ cells and the cecum was enriched for clonally expanded $T_H17$ cells.[84] These data suggest possible roles for cell-extrinsic factors in skewing T-cell phenotypes present in different regions of the gastrointestinal tract. Recently, pathogenic effector $T_H2$ cell clonotypes that were common to both the peripheral blood and esophageal biopsy were detected in patients with EoE.[35] A strategy of analyzing differentially expressed genes of peripheral CD4$^+$ T cells with clonotypes matching those in the tissue against other circulating $T_H2$ clonotypes revealed an upregulation of receptor *GPR15* on esophagus-trafficking clonotypes present in the peripheral blood, providing mechanistic insight into the recruitment of these cells to the esophagus in EoE as well as a method to enrich these cells from the peripheral blood.

A further advantage of scRNA-seq is the ability to simultaneously profile all cell types present in a given sample. Thus, analyses of data from scRNA-seq can also suggest networks of interactions between CD4$^+$ T cells and their microenvironment. A variety of statistical frameworks based on databases of known receptor-ligand interactions have been developed for this purpose.[85-89] A study of lung tissue from patients with or without asthma has suggested that cell-cell interactions in the healthy lung are dominated by tissue-resident memory and tissue migratory CD4$^+$ cells, whereas in the asthmatic lung cell-cell interactions are dominated by $T_H2$ cells engaging in contact-mediated interactions with the epithelium through KLRG1, CD103, and CD49a.[75] A similar analysis revealed that potential axes of communication between pathogenic effector $T_H2$ cells and eosinophils in the esophagus of patients with EoE may include $T_H2$ cytokines and eicosanoid signaling.[35] The IL-18/IL-18R1 axis has been suggested as a mechanism by which inflammatory changes in enterocytes can suppress $T_H17$ cell development and promote Treg cell development in the gut of patients with in ulcerative colitis.[90] A network of cell-cell communication axes constructed from scRNA-seq data collected from lesional and nonlesional tissue of patients with vitiligo has identified that the CCL5/CCR5 axis promoted positioning of Treg cells near CD8$^+$ cells and was required for optimal suppression of CD8$^+$ effector cells by Treg cells in this context.[91]

Although the majority of scRNA-seq–based studies focus on a single disease context, several studies to date have focused on comparing T-cell phenotypes across different tissue or disease contexts. For example, scRNA-seq analysis of T cells recovered from samples across a variety of inflammatory skin pathologies identified signatures of CD4$^+$ T cells that were enriched in leprosy and psoriasis.[22] Two studies have profiled T cells present in both lymphoid and nonlymphoid tissue samples and have established signatures and trajectories associated with adaptation to these distinct tissue niches.[92,93] A pan-cancer T-cell atlas comprising cells from 316 patients with 21 cancer types revealed that tumor-reactive T cells were enriched for TNFRSF9-positive Treg and polyfunctional $T_{FH}/T_H1$ cell phenotypes and suggested that the emergence of these phenotypes are associated with TGF-β and IFN-β signaling present in other T-cell metaclusters.[94] Other studies have sought to establish computational frameworks that enable the projection of single-cell data onto reference data sets, potentially allowing comparison of data sets across a unified transcriptional landscape.[95,96] Efforts to compile, assemble, and integrate scRNA-seq CD4$^+$ T cells recovered from various tissue and disease contexts have the potential to establish the full phenotypic diversity of CD4$^+$ T cells and to enable widespread comparison of markers and signatures associated with different disease states and immune responses.

## FUTURE OUTLOOK FOR scRNA-seq OF CD4$^+$ T CELLS

Single-cell transcriptomics remains a very rapidly advancing technology in academic and commercial settings. Many new single-cell methods aim to enable single-cell multi-omics: the simultaneous acquisition of multiple modalities of data from individual cells, often including whole-transcriptome sequencing. These approaches include cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq), which enables surface protein quantification with DNA-barcoded antibodies, and cell hashing, a related technology that enables the multiplexing of samples and offers greater flexibility in experimental design.[97,98] Specifically, studies of CD4$^+$ cells may aim to adapt this technology to enable assessment of antigen specificity with DNA-barcoded class II tetramer reagents, as has been demonstrated with class I tetramer reagents for CD8$^+$ T cells.[99,100]

Several other approaches for single-cell multi-omics enable the simultaneous analysis of transcriptional states with epigenetic measurements, including chromatin accessibility,[101,102] nucleosome occupancy,[103,104] and DNA methylation.[105,106] These

technologies have helped to pinpoint key epigenetic drivers of $CD4^+$ T-cell differentiation[107] and may help to explore how interactions between transcriptional and epigenetic features of $CD4^+$ T cells affect cellular states and functions. In addition, advances in the feasibility of spatially resolved transcriptomics will help to further answer questions surrounding the behaviors of $CD4^+$ T cells in tissue environments.[108,109] Technology for tracing cellular lineages that maintains compatibility with scRNA-seq (eg, with the use of an evolving clustered regularly interspaced short palindromic repeats [CRISPR] barcode) may enable intraclonal resolution of T-cell lineages, providing unprecedented resolution into the dynamics of $CD4^+$ T-cell differentiation and division.[110-113] These new approaches, in combination with the increasing accumulation of published and publicly available scRNA-seq data, have the potential to further refine our understanding of $CD4^+$ T-cell phenotypes and functionalities and to generate large, multimodal references of T-cell phenotypes across the entire spectrum of human disease.

Recent advances in computational strategies for bioinformatic analysis of antigen-specific TCRs[114-116] and platforms for epitope discovery compatible with $CD4^+$ T cells[117-119] have increased the feasibility of matching antigen-specific TCRs with their specific peptide-HLA epitopes. To date, a major bottleneck in this capability has remained the collection of paired TCR-α/β sequences from clinical samples—especially sparse samples of antigen-enriched $CD4^+$ T cells obtained from the peripheral blood or $CD4^+$ T cells obtained from tissue biopsy samples. Future scRNA-seq studies will likely leverage the capability of scRNA-seq to profile TCR rearrangements to generate large data sets of repertoire data. These data and future advances in repertoire analysis may fuel epitope discovery at a larger scale than previously possible. Improved tools for the integration of single-cell repertoire data with single-cell phenotyping may also provide additional insight into long-standing questions in T-cell immunology about how features of TCRs or epitopes influence the evolution of $CD4^+$ T-cell responses.[120,121] Ultimately, further knowledge of clonotypes that are associated with disease and the epitopes recognized by these clonotypes can be used to inform diagnostics based on public clonotypes or epitopes as well as personalized therapies based on a given patient's antigen-specific repertoire.

Further advances in bioinformatic analysis of scRNA-seq data have the potential to enhance the ability of scRNA-seq to inform clinical applications. Specifically, the development of novel methodologies to generalize results obtained with scRNA-seq to bulk-based sequencing assays may improve the translation of these discoveries to a clinical setting, as bulk sequencing data can be collected from a large number of patients more easily. These methodologies may include methods for the deconvolution of bulk sequencing data, which use scRNA-seq data to precisely define expression profiles associated with single-cell phenotypes and then use these expression profiles to estimate the cell-type composition of a bulk sample.[122,123] In addition, paired TCR-α/β data collected with scRNA-seq can be used to nominate chain pairings for clonotypes in bulk TCR-β sequencing data sets. For example, paired TCR-α/β data generated with scRNA-seq have been used to nominate TCR-α pairings for public peanut-reactive TCR-β sequences detected by using bulk sequencing of TCR-β.[63] These methodologies enable scRNA-seq to be utilized to maximize the biologic insight available from lower resolution but more clinically feasible assays.

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