

Single-cell analysis to understand the diversity of immune cell types that drive disease pathogenesis



Grégory Seumois, PhD, and Pandurangan Vijayanand, MD, PhD La Jolla, Calif

Single-cell next-generation sequencing assays are powerful tools to understand the nature of the immune cells that drive disease pathogenesis. In this brief review we explain the value of performing assays at single-cell resolution to better understand the pathogenesis of allergy, asthma, and other lung diseases. We explain the challenges in performing single-cell studies of airways and lung samples from patients with lung diseases. A major limitation comes from the amount of diseased tissue that can be used for research purposes. Finally, we discuss which sequencing strategies can be used to successfully investigate airway and lung diseases at single-cell resolution. (*J Allergy Clin Immunol* 2019;144:1150-3.)

Key words: Single-cell RNA sequencing, airway disease, Smart-seq2

Genetic variants associated with risk of many lung diseases affect gene expression in a context-dependent manner in a specific subset of immune cell types in the human body.¹⁻⁷ The immune system contains diverse cell types required to defend from infectious agents. At the same time, immune cells can overrespond, resulting in allergy, asthma, and autoimmune and inflammatory diseases. There is probably no other organ system that has a broader influence on human health. However, a complete understanding of the diversity of immune cell types and their properties that help drive disease pathogenesis is still lacking.

Next-generation sequencing (NGS) is one of the newest and most powerful tools to understand biological processes in health and disease. The study of genomes, epigenomes, and transcriptomes provides unbiased information on how genetic variations and environmental signals perturb gene expression and chromatin structure in specific cell types to drive disease

Abbreviations used

NGS: Next-generation sequencing
RNA-seq: RNA sequencing

pathogenesis.^{5,8-14} Genomic assays, such as RNA sequencing (RNA-seq) and microarrays, are routinely performed on a population of cells under the assumption that cells of a particular type are highly similar. However, recent evidence from studies of single cells reveals that this assumption is incorrect; individual cells within the same population can differ dramatically, and these differences might have important consequences for the function of the entire cell population.^{12,15-21}

The application of NGS to the analysis of single-cell transcriptomes and epigenomes has enabled biologists to collect an unprecedented amount of information from each and every cell present in healthy and diseased tissues. The study of individual immune cells in blood, airways, and lung samples from patients with lung diseases (eg, allergy, asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, tuberculosis, and bronchiectasis; Fig 1) will lead to a better definition of the immune cell types driving disease pathogenesis and also help us understand the extent of cellular heterogeneity and molecular plasticity in a “hypothesis-free” manner.^{12,17-20,22-26} These approaches can address significant challenges that currently exist with regard to systematically describing the “molecular status” of pathological or protective cell populations.

Is a dysregulated immune response in the disease state associated with the development of a new cell subset with pathogenic properties or a preferential expansion/contraction of pre-existing subsets of immune cells already physiologically present in blood/lung tissue (Fig 2)? Several studies of chronic diseases in human subjects and model organisms have reported the existence of immune cells that acquire a mixed phenotype. For instance, in patients with latent tuberculosis, T cells responding specifically to *Mycobacterium tuberculosis* antigens display both T_H1 and T_H17 features.^{8,27} Similarly, T_H2 cells with pathogenic properties have been identified from patients with allergy and asthma.^{10,28-31} However, only studies performed at single-cell resolution will enable scientists to determine whether those “disease-specific” functional features are the result of an expansion of a pre-existing population of cells or the result of differentiation in response to environmental signals.^{32,33}

The power of single-cell omics analysis as a discovery tool has been well illustrated in the setting of cancer immunology. Just in the past 2 years, many studies have reported on single-cell omics

From the Division of Vaccine Discovery, La Jolla Institute for Immunology.

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Corresponding author: Grégory Seumois, PhD, Division of Vaccine Discovery, La Jolla Institute for Immunology, 9420 Athena Circle, La Jolla, CA 92037. E-mail: gregory@lji.org.

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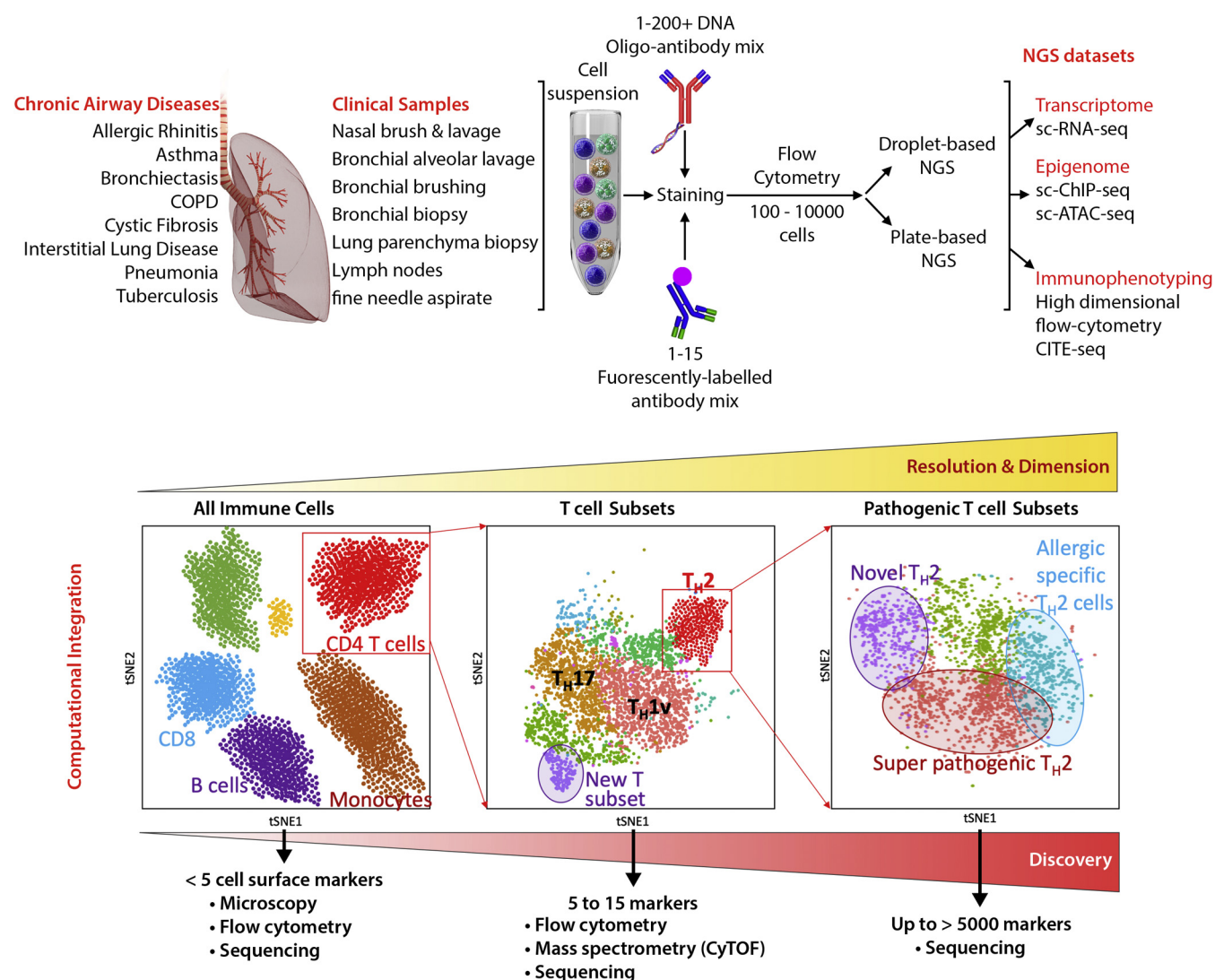


FIG 1. Single-cell NGS using clinical samples from patients with airway and lung diseases. The *top panel* provides an overview of the process. Clinical samples collected from patients will be processed by isolating cellular fraction and stained with both a mix of antibodies recognizing cell-surface markers conjugated with fluorochrome (for flow cytometric analysis) and/or with DNA-barcoded oligos (for immunophenotyping analysis using NGS sequencing). Depending on the number of cells and output data set requested, stained cells will be sorted and processed through 2 single-cell sequencing platform options: the PCR plate-based sorted cells with Smart-seq2 library preparations or the droplet-based 10X platform. The *lower panel* shows the importance of selecting the single-cell sequencing methodology that provides the highest resolution (number of genes per cell). If working with a limited number of cells, the higher the number of genes detected per cell higher will be the clustering resolution and the possibility to separate cells with new molecular features. COPD, Chronic obstructive pulmonary disease; CyTOF, mass cytometry.

analysis of the tumor microenvironment in several human cancer types. These studies have led to the identification of new immune cell subsets, new immune evasion and effector mechanisms, and potentially important molecular targets for cancer immunotherapy.^{23,34-42} For example, our recent single-cell study in patients with lung cancer has led to identification of a highly functional subset of tissue-resident memory T cells, which are likely to be the key cellular targets of anti-programmed cell death protein 1 (PD1) therapy. Furthermore, it has defined a number of novel molecules that are likely to be important for modulating the function of this subset to achieve better antitumor immune responses.^{43,44} Single-cell analysis of tumor samples before and after anti-PD1 treatment have yielded insight into potential

mechanisms and biomarkers that can distinguish responders from nonresponders to immunotherapy.^{43,45} Studies in the cancer field highlight that single-cell analyses of diseased tissue samples can provide information relating to key pathogenic mechanisms driving disease, as well as help us understand the mechanisms of action of novel interventions.

The relatively easy access to excess tumor tissue samples from standard diagnostic or therapeutic procedures has enabled research to rapidly conduct these single-cell studies in large numbers of cells and patient samples. However, for patients with lung diseases, such as asthma and chronic obstructive pulmonary disease, it is not routine clinical practice to obtain airway tissue samples. This issue, coupled with the limited amount of tissue that

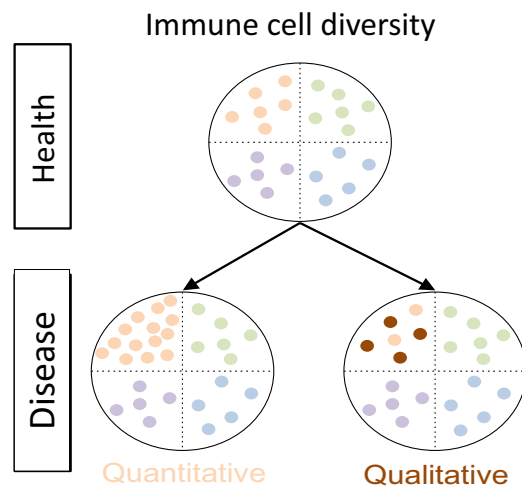


FIG 2. Schematic representation of the information provided by using single-cell analysis. The *top panel* shows the distribution of 4 immune cell subsets in a healthy subject. The *bottom panel* shows the possibilities that can happen in disease states: the relative proportion of one cell subset is increased without changes in the molecular program (quantitative change) and/or cells in one subset change their molecular program (qualitative change); such differences can be captured by using single-cell analyses.

can be obtained for research purposes, might explain the relative paucity of single-cell studies in patients with lung diseases. One way to address this limitation is to study lung diseases in model organisms. For instance, single-cell analysis of the development of tracheal epithelial cells *in vivo* has led to identification of new cell types and a better understanding of cell lineages in the context of pulmonary fibrosis.⁴⁶ More recently, in a mouse model of asthma driven by house dust mite allergen exposure, Tibitt et al⁴⁷ showed that the repertoire of T helper cells in inflamed lung tissue is much more heterogeneous than usually accepted. However, airway and lung diseases are long-lasting diseases, and mouse models, by definition, capture only part of the complex human biology. Thus human studies are likely to provide deeper insight into disease pathology.

An important technical limitation for studying human tissue samples with low cell numbers is that the most commonly used high-throughput platforms for single-cell analysis require several thousand cells as input material. Samples with just tens to hundreds of cells would require a different approach that relies on single-cell sorting by using flow cytometry into 96-well plates after a single-cell library preparation (Fig 1, upper panel). By far the most successful method is the optimized single-cell RNA-seq library preparation adapted from the Smart-seq2 protocol (also commercially developed by Takara [Mountain View, Calif], ICe18).^{48–51} When combined with index sorting, high-dimensional protein expression data can also be obtained in parallel.⁵² This method, although laborious when prepared manually, allows researchers to review the complete surface phenotype of every single cell sorted into a plate and associate that event with its transcriptome. The other method is the droplet-based single-cell RNA-seq assay,^{53,54} as commercialized by 10X Genomics (Pleasanton, Calif). As shown in a comparative study,⁵⁵ Drop-seq assays generate a lower-resolution data set, making computational analysis difficult, especially when analyzing highly similar cell subsets, such as T-cell subsets, as opposed to more diverse cell types, such as PBMCs (Fig 1, lower

panel). Our analysis showed that the Smart-seq2 assay detected twice the number of genes compared with the commercial Drop-seq assay (10X Genomics).⁵⁶ At the level of individual genes, the sensitivity of both assays was similar for highly expressed genes, such as *B2M*. However, for several genes of interest, only 30% to 40% of cells showed expression using 10X Genomics, whereas 85% to 95% of cells showed expression when using the Smart-seq2 assay.⁵⁶ This contrast is more important when working with resting immune cells, such as T cells, that contain a much lesser amount of RNA.

In early 2019, 10X Genomics released a newer version with a greater rate of mRNA capture (resolution). This assay, when coupled with sample multiplexing using DNA oligo-barcoded antibodies⁵⁷ or DNA oligo-barcoded lipid anchoring in cell membrane,⁵⁸ will enable the study of airway and lung samples at much greater resolution in a cost-effective manner.

Recently, NGS assays that profile the epigenetic state of cells are also available for studying clinical samples with limited cell numbers. Such single-cell epigenetic methods include histone modification profiling by using immunoprecipitation followed by sequencing (sc-ChIP-seq)^{59,60} or the more accessible and cost-effective profiling of the Assay for Transposase Accessible Chromatin with high-throughput sequencing (sc-ATAC-seq).⁶¹ These assays will complete the arsenal of tools required for understanding cellular heterogeneity and the mechanisms of cellular differentiation or activation in response to pathogenic perturbations.⁶²

In summary, single-cell high-dimensional immune phenotyping, RNA-sequencing, and chromatin accessibility profiling analyses of immune cells isolated from blood, airway, and lung tissue samples from patients with lung diseases will provide insight into the molecular and cellular mechanisms that are driving health and disease states. These approaches are likely to constitute the cardinal measures for future development of diagnostic tools, novel biomarkers, and therapeutic interventions in the emerging field of precision genomic medicine.

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