Applications of single-cell sequencing in upper and lower airway diseases: progress and perspectives

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Abstract

Single-cell sequencing (SCS), including genomic, transcriptomic, epigenomic, proteomic, and metabolomic sequencing, is a powerful tool for revealing cellular and molecular landscapes at the level of single-cell resolution. The use of SCS in upper and lower airway diseases has revealed numerous intrinsic biological characteristics and dynamics of airway inflammation, cancer, viral infection, and other lesions, and has also constructed an immune landscape of health and disease states. In this report, we review how advances in SCS technology have increased our understanding of upper and lower airway diseases, including chronic rhinosinusitis, allergic rhinitis, asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, and other inflammatory diseases, as well as the landscape of airway immune cells in healthy and pathological conditions. We also discuss the potential mechanisms of tumor heterogeneity, circulating tumor cells, and tumor biological behavior. The rapid development of single-cell technology will further increase our understanding of the biological characteristics of upper and lower airway diseases and provide precise treatment targets for patients.

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Abstract

Single-cell sequencing (SCS), including genomic, transcriptomic, epigenomic, proteomic, and metabolomic sequencing, is a powerful tool for revealing cellular and molecular landscapes at the level of single-cell resolution. The use of SCS in upper and lower airway diseases has revealed numerous intrinsic biological characteristics and dynamics of airway inflammation, cancer, viral infection, and other lesions, and has also constructed an immune landscape of health and disease states. In this report, we review how advances in SCS technology have increased our understanding of upper and lower airway diseases, including chronic rhinosinusitis, allergic rhinitis, asthma, chronic obstructive pulmonary disease, pulmonary fibrosis, and other inflammatory diseases, as well as the landscape of airway immune cells in healthy and pathological conditions. We also discuss the potential mechanisms of tumor heterogeneity, circulating tumor cells, and tumor biological behavior. The rapid development of single-cell technology will further increase our understanding of the biological characteristics of upper and lower airway diseases and provide precise treatment targets for patients.

Key words: Upper and lower airway; Single-cell sequencing; Single cell map of the airway; Immune microenvironment; Tumor circulating cells.

Abbreviation

SCS	Single-cell sequencing
scDNA-seq	genome sequencing
scRNA-seq	transcriptome sequencing
scATAC-seq	DNA methylation sequencing, histone modification sequencing, chromatin structure sequencing
AR	allergic rhinitis
BA	bronchial asthma
COPD	chronic obstructive pulmonary disease
IPF	idiopathic pulmonary fibrosis
TJ	tight junctions
eCRSwNP	eosinophilic CRS with nasal polyps
MCs	Mast cells
CRSwNP	chronic rhinosinusitis with nasal polyposis
MCTC	tryptase and chymase
MCT	tryptase without chymase
AERD	aspirin exacerbating respiratory disease
iMCT and iMCTC	inflammatory MCT and MCTC
HDM	house dust mite

ILC2	type 2 congenital lymphocytes
LUAD	lung adenocarcinoma
AT2	alveolar epithelial type II
PBMCs	peripheral blood mononuclear cells
ceRNA	competing endogenous RNA
PPI	protein–protein interaction
AT1	alveolar type 1 cell
AT2	alveolar type 2 cell
MMP7	matrix metalloproteinase 7
EMT	epithelial-mesenchymal transition
SPP1	the lesion secrete a type 1 phosphorylated protein
COVID-2019	coronavirus disease 2019
BALF	paired bronchoalveolar lavage fluid
DCs	dendritic cells
MDSCs	myeloid suppressor cells
MP	megakaryocyte progenitor
IFN-I	the type I interferon
MAPK	mitogen activated protein kinase
NPC	Nasopharyngeal carcinoma
CAFs	stromal cells was fibroblasts
NK cells	natural killer cells
NSCLC	non-small cell lung cancer

Introduction

Single-cell sequencing (SCS) is an emerging technology used to understand the function and gene expression status of individual cells, as well as interactions that occur at the single-cell level by use of single-cell suspensions, preparations, single-cell capture and labeling methods, library preparation and sequencing, and data analysis [1]. Currently, SCS technologies have been used in genome sequencing (scDNA-seq), transcriptome sequencing (scRNA-seq), DNA methylation sequencing, histone modification sequencing, and chromatin structure sequencing (scATAC-seq). SCS studies conducted on heterogeneous diseases have demonstrated significant advantages when compared to studies conducted using traditional sequencing methods [1, 2]. For instance, SCS technology has been widely used in the field of upper and lower airway diseases, such as inflammatory diseases, viral infections, and cancers [3]. Moreover, it can be used to identify rare subgroups, circulating inflammatory cells, tumor or immune microenvironments, cancer stem cells, and to classifry the heterogeneity and molecular subtypes of airway inflammations and tumors[4]. Futhermore, SCS can be used to study various mechanisms associated with disease onset, progression, tumor metastasis, evolution, relapse, and treatment resistance. Here we provide a systematic review of current advances in the application of several common SCS techniques in the upper and lower airways.

1. SCS in airway inflammatory diseases

The upper and lower inflammatory airway diseases discussed in this review are allergic rhinitis (AR), chronic rhinosinusitis (CRS), bronchial asthma (BA), chronic obstructive pulmonary disease (COPD), and idiopathic pulmonary fibrosis (IPF). These diseases are characterized by pathological features such as luminal inflammation, epithelial chemosis and hyperplasia of the basement membrane, smooth muscle and submucosal cells, as well as glandular hyperplasia. Their common features are epithelial barrier dysfunction, including defective tight junctions (TJs) between cells and increased epithelial permeability. ScRNA-seq is widely used in the field of immune diseases due to its advantages of high cell throughput, low cost of library construction, and short capture cycle. At present, scRNA-seq studies of upper and lower airway diseases have revealed that the airway has a complex structure and contains multiple cell types, such as epithelial cells that act as

a barrier, and immune cells for antimicrobial defense. ScRNA-seq is also used to understand airway biology, disease pathogenesis, and disease progression between the upper and lower inflammatory airways. Here, we discuss the use of single cell sequencing in several different airway inflammatory diseases on individual basis.

1.1 Allergic rhinitis

AR is a disease with a high worldwide prevalence and is caused by common allergens encounterd in daily life, such as pollen, molds, and dust mites. AR causes symptoms such as sneezing, runny nose, nasal congestion, and itching, and also involves mucositis [5]. It is currently believed that pathogenic memory Th2 cells are important driving factors during the sensitization stage of AR [6]. Recent scRNA-seq studies of pathogenic cells and molecular mechanisms have revealed the complex heterogeneity cell types involved in AR. ScRNA-seq showed that monocytes are recruited to the nasal mucosa within hours of a local allergen attack, and macrophages carrying genes for Th2-related chemokines are highly expressed and have an activation phenotype driven by both IL-4 and IL-13 [7]. In addition to the increases in Th2 chemokines in ARstates, the levels of Th17 and Treg were also found to be significantly upregulated. The expression levels of Th17 related genes (*S100A12* , *PI3* , *DEFB4A*) and histamine receptors (HRH1 and HRH2) were upregulated in the nasal mucosa of AR patients [8]. Su et al. [9] used scRNA-seq to conduct a clinical case-control study of 600 Chinese AR patients by extracting DNA from blood samples and found that polymorphisms in the *TNFSF4* and*BLK* genes may be associated with susceptibility to AR in the Han Chinese population. Therefore, whether single cell sequencing can be used to further elucidate the complex gene environment interactions in AR will be the next challenge. Considering that AR has multiple clinical (phenotypic) and mechanistic (endotypic) forms, we can also look forward to current scRNA-seq methods providing better stratified therapeutic strategies based on detailed clinical and molecular diagnostics.

1.2 Chronic rhinosinusitis (CRS)

CRS is a chronic inflammatory disease that manifests with symptoms such as nasal congestion, hyposmia, and facial pain occurring in the mucosa of the sinuses. CRS has a complex pathogenesis, and some patients do not respond well to systemic medications and surgery. According to epidemiological data, approximately 8% of all people have CRS, among which chronic rhinosinusitis with nasal polyps (CRSwNP) accounts for 25% to 30% [10].

Due to the rapid development of updated single-cell sequencing technology, our understanding of CRS has progessed to studying various pathways involved in its pathogenesis. ScDNA-seq revealed that B cells differentiate into IgE-producing B cells via IgG/IgA1-IgE class switching, which induces an allergic reaction involving nasal bacterial protective mucosa in CRSwNP patients [11]. In addition, epithelial cell dysfunction, abnormal stem cell differentiation, and epithelial cell remodeling are also important reasons for the occurrence of type 2 CRSwNP. A Pistochini et al. [12] used scRNA-seq technology to validate genes that may be involved in the inflammatory mechanism of CRS, and unexpectedly found that *AQP5* , *CAV1* , *LTF* , and *MGB1* gene expression, which is potentially associated with epithelial dysfunction, was significantly reduced in nasal polyp patients. In 2018, researchers used the Seq Well platform to analyze the ethmoid sinus of patients across the CRS spectrum, and described the types of human respiratory epithelial cells, immune cells, stromal cells, as well as the transcriptome of subtype 2 inflammatory diseases. That was the first time that researchers found that the abnormal differentiation tracks of stromal cells in nasal polyps led to changes in epithelial cell diversity [13]. A recent study of RNA sequencing cell type transcriptional characteristics data obtained from patients by nasal sinus epithelial scrubbing found that airway epithelial remodeling in nasal polyps was caused by cell type changes and proglandin E2 (PGE2) [14]. The above research on epithelial cells in CRS has paved the way for revealng the profile of the CRS endogenous transcriptome. Finally, a single cell level analysis of immune cells and non-immune cells in healthy individuals and heterogeneous CRS patients revealed that of the many specific cell subsets and molecules, ALOX15+ macrophages recruit eosinophils, monocytes and Th 2 cells to participate in the pathogenesis of eosinophilic CRS with nasal polyps (eCRSwNP) type 2 immunity by secreting chemokines [15]. Mast cells (MCs), as sentinels of the immune system, infiltrate different MC subsets in type 2 airway allergic inflammatory diseases. These infiltrated MCs include subepithelial MCs expressing the proteases tryptase and chymase (MCTC) and epithelial MCs

expressing tryptase without chymase (MCT). Daniel F. Dwyer [16] used scRNA seq to perform a MC analsyis of CRWwNP, and identified a CD38high CD17high intermediate state cell. Furthermore, the numbers of those intermediate state cells were significantly increased in CRSwNP patients with aspirin exacerbating respiratory disease (AERD). Those investigators first proposed that the expanded mucosal T2-inflammation-associated MC subsets be reclassified as airway inflammatory MCT and MCTC (iMCT and iMCTC). The proposed existence of an intermediate MC subtype is now crucial for the treatment of T2 type CRSwNP with different severity levels. Thus, for therapeutic interventions in polyposis, it may be possible to ameliorate the development of CRS by blocking or inhibiting key MC genes with monoclonal antibodies, and thereby altering MC proliferation and transcriptional activity.

It goes without saying that SCS analysis of CRS provides strong theoretical support for a further in-depth exploration CRS pathogenesis and cell specific mechanisms.

1.3 Asthma

Asthma is considered as a respiratory disease characterized by chronic airway inflammation that involves a variety of cells (eosinophils, mast cells, T lymphocytes, neutrophils, airway epithelial cells) and cellular components. It is associated with airway hyper-reactivity, and usually with widespread and variable reversible expiratory airflow restriction which can lead to recurrent symptoms such as wheezing, shortness of breath, chest tightness and cough, with the intensity varying over time [17]. The phenotype of asthma can be divided into exogenous, endogenous and mixed asthma based on different clinical manifestations, pathophysiological characteristics, and prognostic differences [18]. The interaction of inflammatory cells with the external environment results in airway inflammation, airflow restriction, hyperreactivity, and airway remodeling during the pathologic progression of asthma. At present, the pathogenesis of asthma is not clearly defined.

The current applications of SCS in asthma have mainly focused on immune cells and lung epithelial cells involved in its pathogenesis. Researchers have used scRNAseq to analyze subsets of lung immune cells in an asthma mouse model induced by the house dust mite (HDM). The results identified 20 immune subsets and revealed that basophils, type 2 congenital lymphocytes (ILC2), and CD8+specific cell clusters Memory T cells were the main sources of IL-4 and IL-13 [19, 20]. Subsequent studies used scRNA-seq and scATAC-seq to sequence alveolar lavage fluid from asthma patients and found that the Foxp3+Treg, Th1, Th2, and Th17 cell subpopulations were significantly increased, and the characteristic genes of Th2 cells were mainly *Cd200r1* , *Il6* , *Plac8* , and *Igfbp7* . Moreover, a differentiation disorder of T cells was found to lead to a Th1/Th2 imbalance, and the development and occurrence of asthma [21]. As antigen-presenting cells, DCs play an important role in the allergen-driven Th2 immune response in asthmatic airways. Gentaro Izumi et al. [22] identified 5 distinct lung conventional CD11b+ DCs (cDC2) clusters by use of scRNA-Seq technology, and found that lung cDC2 promoted Th17 and Th2 differentiation at different maturation stages. Li et al. [23] used scRNA-seq to show that neutrophils promoted the uptake of allergens by lung CD11b+Ly-6C+DC and increased susceptibility to exacerbated asthma in the lung tissues of asthmatic mice. The key immune cell phenotypes of patients with asthma exacerbation mainly include monocytes, CD8+T cells and macrophages, and investigators have clearly proposed candidate genes that are closely related to asthma exacerbation [24]. Lung epithelial cells are important for maintaining stable lung structure. ScRNA-seq revealed that IL-1β produced by human airway epithelial cells increases airway mucus concentrations by inducing HAEC to release MUC5B, leading to airway remodeling and pathogenic mucus hypersecretion in asthma [25]. Subsequent studies discovered a new type of ciliated epithelial cell related to asthma, and proved that epithelial cell dysfunction with increased goblet cell and mucus production was the initial pathologic factor in asthma [26]. SCS studies of asthma have provided new insights into the role of epithelial and immune cell function in epithelial cell remodeling in asthma.

1.4 COPD

COPD is the leading cause of respiratory death worldwide, and is also closely related to heart disease [27]. COPD is characterized by its persistent symptoms and destruction of lung parenchymal tissue, and especially during its advanced stages [28]. The cell-specific mechanisms underlying the pathobiology of COPD are

not fully understood. ScRNA-seq has been used to obtain single-cell resolution and identify changes in COPD mechanisms, cell phenotypes, and alveolar niche crosstalk [29, 30]. Maor et al. [30] constructed single-cell RNA sequencing profiles of explants from subjects with advanced COPD or control lungs. Those profiles focused on three cell types associated with COPD pathogenesis: epithelial cells, endothelial cells, and alveolar macrophages. The profiles identified 33 abnormal types of basal cells, and identified the CXCL signal transduction that originates from endothelial cells via a network analysis, and found the common cell type specific transcriptional aberrations, and revealed that alveolar macrophages overexpressed metallothionein and HMOX1.

Recently, researchers analysis of 70,030 epithelial cells of lung adenocarcinoma (LUAD) and normal origin through single-cell RNA sequencing have identified the alveolar epithelial type II (AT2) cell subsets that express HHIP, and found that the capillary CXCL motif chemokine signal is an important cause of COPD alveolar inflammation. Another conclusion was that the alveolar macrophage subgroup of metallothionein is enriched in COPD, which provides a more profound understanding of COPD pathophysiology [31, 32]. At the same time, scRNA-seq was used to identify cell-specific differences and changes in individual protein levels that may contribute to the development of emphysema in severe COPD, More than 20,000 cells were assessed for cell type markers and cell-specific gene expression characteristics, from which IGFBP5 and QKI were identified as ciliated epithelial genes associated with severe COPD [33]. Based on the long-standing consensus that the immune response primarily mediates the pathology of chronic obstructive pulmonary disease (COPD), and that exosomes may be involved in the immune regulation of COPD, Research Scholars sequenced plasma exosomes and performed single-cell RNA sequencing on peripheral blood mononuclear cells (PBMCs) from patients with COPD and healthy controls,they identified 135 mRNAs, 132 lncRNAs, and 359 circRNAs from exosomes that were differentially expressed in six patients with COPD compared with four healthy controls, constructed competing endogenous RNA (ceRNA) and protein–protein interaction (PPI) networks to delineate the interactions between PBMCs and exosomes within COPD which has provided a more specific pathological process of COPD by enabling a series of data analyses of biological information, and constantly integrating cell types and their interactions.[34]

1.5 IPF

IPF is a deadly respiratory disease that mainly occurs in middle-aged and elderly people [35]. It is an interstitial disease in which fibroblast expansion occurs in parallel with excessive production and deposition of extracellular matrix, leading to airway remodeling, inflammation, alveolar destruction, and fibrosis [36]. Currently, many scRNA-seq studies have provided new insights into the cell types and cell interaction-related biological processes involved in IPF pathogenesis. Barry R Strip et al. [37] mapped epithelial cell types in normal and IPF airways by scRNA-seq and revealed the reconstruction of IPF basal cell diversity. Taylor S. Adams et al. [38] constructed a single-cell atlas of IPF that focued on aberrant epithelial, fibroblast,and endothelial cell populations, and identified all known lung epithelial cell populations in control samples, including alveolar type 1 (AT1) and type 2 (AT2) cells, ciliated cells, basal cells, goblet cells, club cells, pulmonary neuroendocrine cells, ionocytes, and 80 aberrant basaloid cells. The occurrence of IPF is closely related to alveolar epithelial injury. ScRNA-seq of IPF lung tissues revealed that matrix metalloproteinase 7 (MMP7), integrin αVβ6, cellular senescence, and epithelial-mesenchymal transition (EMT) abnormal epithelial cells were highly expressed at the edges of fibroblastic lesions, whereas they were not present in normal lung tissues, which once again suggests that the development of IPF is closely related to alveolar epithelial damage. This is because dysfunctional epithelial cells interact with mesenchymal cells, immune cells, and endothelial cells via various signaling mechanisms which trigger the activation of fibroblasts and myofibroblasts, whereas abnormal NOTCH2+ basal cells are susceptible to metabolic dysfunction, aberrant epithelial activation, and epithelial repair dysfunction [39]. Fibroblasts are the central mediators of extracellular matrix production in IPF. Rebecca Peyser et al. [40] identified 49 signature genes and delineated fibroblast subpopulations by scRNA-seq techniques. They found that fibroblast activation was poorly correlated with the expression of transforming growth factor-β pathway genes. The immune microenvironment involved in IPF has now been revealed by use of scRNA-seq technology. Those studies showed that macrophages around the lesion secrete a type 1 phosphorylated protein (SPP1) that causes organ fibrosis, and that the T cells

involved in anti-fibrotic and pro-fibrotic activates are mainly Th9, Th22, and γδT cells [37]. It is self-evident that fibroblasts, epithelial cells, and immune cells do not independently influence the formation and development of IPF. A combination of biological approaches, as well as *in vivo* modeling will provide important data regarding the transcriptomic changes and cellular interactions associated with IPF development, and those data will need to be further validated by measurements of gene and protein expression.

2. Use of SCS in airway viral infectious disease

In the challenging environment of coronavirus disease 2019 (COVID-19), SCS was positioned to help researchers understand immune cell subsets and molecular factors associated with protective or pathological immunity against the severe acute novel coronavirus infection respiratory syndrome, and then develop vaccines and targeted therapies. Xu et al [41] used early scRNA-seq methods to characterize peripheral blood mononuclear cells (PBMCS) and paired bronchoalveolar lavage fluid (BALF) cells from non-infected control subjects and COVID-19 patients, and subsequently reveal the different immune responses to COVID-19. Valuable findings were as follows: (1) decreased numbers of dendritic cells (DCs) were associated with increased numbers of mononuclear cells such as myeloid suppressor cells (MDSCs). (2) When compared with the healthy control subjects, the numbers of peripheral T cells and NK cells were significantly decreased in severe COVID-19 patients, and especially the innate T cells and various CD8+ T cell subsets (3). In severe patients, the proportions of various activated CD4+ T cell subsets (including Th1, Th2, and Th17-like cells) in T cell compartments were increased with increased clonal amplification [41, 42]. To elucidate peripheral immune cellular pathways that may lead to immunopathology or protective immunity in patients with severe COVID-19, PBMCS were collected from seven hospitalized patients with severe COVID-19 and analyzed using scRNA-seq and peripheral immune cell phenotype information, Class II HLA downregulation. The developing neutrophils characterized by heterogeneous interferon-stimulating genes in COVID-19 patients were identified [43].

To further explore the pathogenesis of COVID-19 and identify precise therapeutic targets, the COVID-19 Single Cell Consortium of China (SC4) generated a scRNA-seq dataset consisting of 171 COVID-19 patients, and integrated the basic characteristics of the dataset and cell subsets in different major cell lineages. As determined by SCS, the severity of COVID-19 was associated with airway epithelial-immune cell interactions. Huang et al. [44] revealed the dynamic changes in blood immune response in COVID-19 patients at different stages by use of gene expression detection, as well as T cell receptor (TCR) and B cell receptor (BCR) transcriptome analyses. In the peripheral blood of patients with severe COVID-19, the levels of MKI67 in plasma cells were increased, and the levels of plasmacytoid dendritic cell cluster DC-C4-LILRA4 were decreased during both the progression and recovery stage. After adjusting for technical covariates, the Neu-c3-CST7 neutrophil cluster was found to be associated with patient age, COVID-19 severity, and disease stage, while for T cells, a subgroup with high MKI67 expression was closely related to COVID-19 severity. The scRNA seq data for 341420 PBMCS, 185430 cloned T cells, and 28802 cloned B cells were obtained from 25 samples from 16 patients with COVID-19. It was found that the numbers of DCs, CD14+Monocyte and megakaryocyte progenitor (MP) cells, and CD8+T lymphocytes in severe patients were significantly reduced, and the type I interferon (IFN-I), mitogen activated protein kinase (MAPK) and ferroptosis pathways were activated during disease activity, but then gradually recovered after the patient's condition improved [45]. The scientific community has made a bold attempt to use new algorithms in scRNA-seq to discover and summarize how innate and adaptive immune cell subsets and immune factors are related to the development of COVID-19 vaccines and other forms of treatment [46]. For example, 21 published single-cell sequencing datasets (totaling > 3.2 million cells) now provide a comprehensive study and meta-analysis of the immunology of severe acute respiratory syndrome coronavirus type 2 infection. Researchers have identified putative targets of the severe acute respiratory syndrome coronavirus in tissue-resident cell populations. Type II lung cells, ileal absorptive intestinal cells, and nasal cup secretory cells were found to co-express ACE2 and TMPRSS2, while the host protease (TMPRSS2) cell coronavirus binds to the stinger protein of the type 2 virus to facilitate its entry into the cell [47]. Overall, in terms of cellular variants, the proportions of circulating plasma cells and classical monocytes are dramatically increased in COVID-19 patients, while the proportions of DCs, non-classical monocytes, NK cells. and some lung progenitor cells are

decreased significantly, decreased. With regards to genetic variants, patients showed a significant increase in*AREG* , *EREG* , and *HLA* class II genes, as well as in genes related to the "IL-17 signaling pathway," "response to toxic substances,""lymphocyte/T cell activation," and "positive regulation of immune effector processes" [48]

In short, various single-cell transcriptome sequencing studies on COVID-19 have achieved precise results. Although the epidemic has been under relatively stable control after a three-year run, further tracking and intervention in the sequelae of COVID-19 by use of single-cell techniques will bring new opportunities and challenges.

3. Use of SCS in airway tumors

In 2011, SCS was first applied to human cancer cells to detect cellular and microenvironmental heterogeneity at high resolution [49]. It was also widely used to detect key signature genes during tumor progression [50]. The development of SCS has led to breakthroughs in our understanding of tumor heterogeneity, the tumor microenvironment, immune checkpoints, intercellular communication lattices, and targets for relapse therapy in airway tumors [51-53]. It is an important tool for exploring interactions between tumor cells and neighboring stromal cells and immune cells. Currently, single cell sequencing is used in nasopharyngeal, laryngeal, and lung cancers.

3.1 Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is a genetically related squamous cell carcinoma of nasopharyngeal epithelial origin with a different immune infiltration and a lower degree of differentiation than other carcinomas. It occurs mainly in Southeast Asia and North Africa [54]. Clinical observations have shown that malignantly transformed nasopharyngeal epithelial cells are often mixed with large numbers of stromal cells. Lanqi Gong et al. used scRNA seq to confirm the presence of large numbers of anisotropic stromal cells in malignant tumors, and discovered that an important component of stromal cells was fibroblasts (CAFs), which can alter the immune microenvironment by inhibiting the activity of immune effector cells and recruiting immunosuppressive cells. The immune microenvironment can be altered by inhibiting the activity of immune effector cells and recruiting immunosuppressive cells, thereby enabling cancer cells to evade immune surveillance [55-57]. The tumor microenvironment in NPC predominantly undergoes changes in T cells and B cells. Five major stromal populations and 36 different subpopulations were identified by use of scRNA-seq combined with the clonal identification of T and B cells, and the results were further verified by constructing a map of nasopharyngeal carcinoma infiltration into stromal cells. The map showed that the CD45+ immune cells included T cells, B cells, natural killer cells (NK cells), and myeloid cells, with the T cells being mainly characterized by a significant up-regulation of *CXCL1* 3 and *LGALS1* gene expression. The differentially expressed genes for B cells were mainly interferon-induced and immunoglobulin-encoded, including *IFITM1* ,*IFI44L* , *IGHA1* , *IGKC* , and *IGHG* . Both the T and B cells were affected by IFN-γ and IFN-α over-release [58]. In terms of cellular interactions, three clusters of depleted T cells (HAVCR2, TOX, and LAG3) were found to preferentially interact with memory B cells, innate-like B cells, inactivated B cells, and IFN-induced B cells in NPC [59, 60]. These studies elucidated the degree of tumor heterogeneity and the intercellular network of NPC at the level of single-cell resolution. They also provided insights into the mechanisms of NPC progression and suggested strategies for developing precise therapies for NPC.

3.2 Laryngeal cancer

Laryngeal cancer is closely related to the human papillomaviruses HPV16 and HPV18. It occurs in laryngeal mucosal epithelial tissue, where is causes a foreign body sensation in the throat, discomfort when swallowing, pain in the throat, or an irritating cough accompanied by blood in the sputum [61]. Song et al. [62] divided laryngeal tumor tissue cells into categories of tumor cells, immune cells, epithelial cells, fibroblasts, and endothelial cells by performing single-cell sequencing. The data showed that keratinocyte-like tumor cells highly expressed *SPRR3* , *SPRR1B* , and *SPRR1A* , which are members of the SPRR family, and encode the specific markers for keratinocyte and squamous epithelial cell differentiation. SCS showed that SLAMF9 was specifically expressed in the non-immune cells of a rare form of laryngeal chondrosarcoma, and this

finding was verified by immunohistochemistry [63]. Wang et al. [64] selected three prognosis-related IRLs (BARX1-DT, KLHL7-DT, and LINC02154) by the machine learning method, and used them to predict the prognosis, immune infiltration status, and immunotherapy response of LSCC patients.

3.3 Lung cancer

Lung cancer is a highly malignant tumor of the bronchial mucosa or glands in the lung and displays rapid growth and early metastasis. Current SCS studies have revealed a central role played by cytotoxic and effector T cells, NK cells, and different functional macrophage (Mφ) subtypes in the immune microenvironmental heterogeneity of lung adenocarcinoma and lung squamous cell carcinoma [65]. Wu et al. [66] analyzed 42 tissue biopsies from patients with stage III/IV non-small cell lung cancer (NSCLC). Those analyses identified the rare cell types of follicular dendritic cells and T helper 17 cells in tumors, and explained the correlation between tumor heterogeneity and tumor-associated neutrophils. Li et al. [67] identified a cancer cell subtype that deviated from the normal differentiation trajectory and dominated the metastatic stage. The cells were obtained from 208,506 cells in normal tissue or from 44 patients with early-stage to metastatic cancer. The investigators found that the normal resident myeloid cell population was gradually replaced by monocyte-derived macrophages and dendritic cells, and was accompanied by T-cell failure. Overall, although there are still batch effects and difficulties with clinical implementation, SCS has provided new insights into the precision and accuracy of molecular cancer research on the tumor microenvironment and cell heterogeneity, and has significantly improved our understanding of cancer diagnostic stratification, biomarkers, precision therapy, and prognosis.

4. Conclusion

SCS has revealed some important molecular mechanisms of airway physiology and pathogenesis of inflammatory diseases, cancer, and viral sensitization (summarized in Table 1 and Figure 1). It has also suggested new research perspectives for subsequent studies. Regarding inflammatory diseases of the upper and lower airways, the immune microenvironment, different immune cell subpopulations, and inflammatory heterogeneity mechanisms have been explored, and regulation of the immune response by the airway epithelial cell barrier has been described by application of SCS technology. With regards to airway viral infections and tumorigenesis, a large amount of data is now available to construct a cellular atlas that more comprehensively describes the cellular atlas of normal human lung tissue. RNA-Seq analysis studies are now advancing not only within single cells but also in a variety of cells and organs by using molecular information with high spatial and temporal precision. Of course, SCS has some limitations; for example the accurate transcription rates of RNA-Seq may not be consistent across all eukaryotic cells. Additionally, cell isolation procedures, including upstream and downstream processing, vary widely across studies, which limits reproducibility and affects the accuracy of reported datasets, often leading to over- or under-reporting of specific cell subpopulations. Future single-cell multi-omics technologies will be geared toward a broader range of airway diseases, and more accurately reveal the specific mechanisms of action of multiple cell types, and even subtypes in airway diseases. In addition, we believe that future efforts may also focus on applying SCS technology to a wide range of airway diseases, such as the correlation between AR and asthma development, NPC metastasis, disease recurrence, drug resistance, and phylogeny, in order to better understand the origins, treatments, and proper care of patients with airway diseases.

Table 1 Important findings regarding airway diseases achieved using SCS

Airway Diseases	Platform	Sample Source	Main findings
AR	Microarray	Human nasal mucosa	Described the gene expressio
	NovaSeq	PBMCs	Single-cell immunoassays afte
CRS	10X	Nasal irrigation solution	Cellular stress responses indi
	10X	Human nasal polyps	GATA3 expression is a featu
Asthma	10X	Mouse lung tissue	MiR-155 deficiency altered lu
	10x	Monkey bronchial epithelium and lung tissue	An analysis of intercellular in
COPD	Illumina NextSeq 500	Human alveolar lavage fluid	Coexistence of monocyte-deri

	10X	Human lung tissue	IGFBP5 and QKI as Identifi
	Drop-seq	Human small airway epithelium	Identified novel cell-specific e
IPF	10X	Human lung tissue	Provided a single-cell atlas of
	10X	Mouse lung tissue fragments	Reports on the development
	10X	Human lung tissue	Describes the proliferation of
	Illumina NextSeq 500	Human lung tissue	Shows that NOTCH2 restrict
COVID-19	Drop-seq	Human lung epithelial cells	Uses airway epithelial cells to
	inDrops	Mouse bronchial epithelium	Single-cell mapping of airway
	10X	Human lung epithelial cells	An immunophenotypic analys
NPC	Illumina NovaSeq 6000	Human nasopharyngeal carcinoma tissue	Highly representative features

Figure 1:The left side of the figure depicts a brief procedure for on-line analysis of simple samples for single-cell transcriptome sequencing, The right side mainly shows the application of SCS in airway inflammatory diseases, viral infections, and cancer diseases and major research findings.

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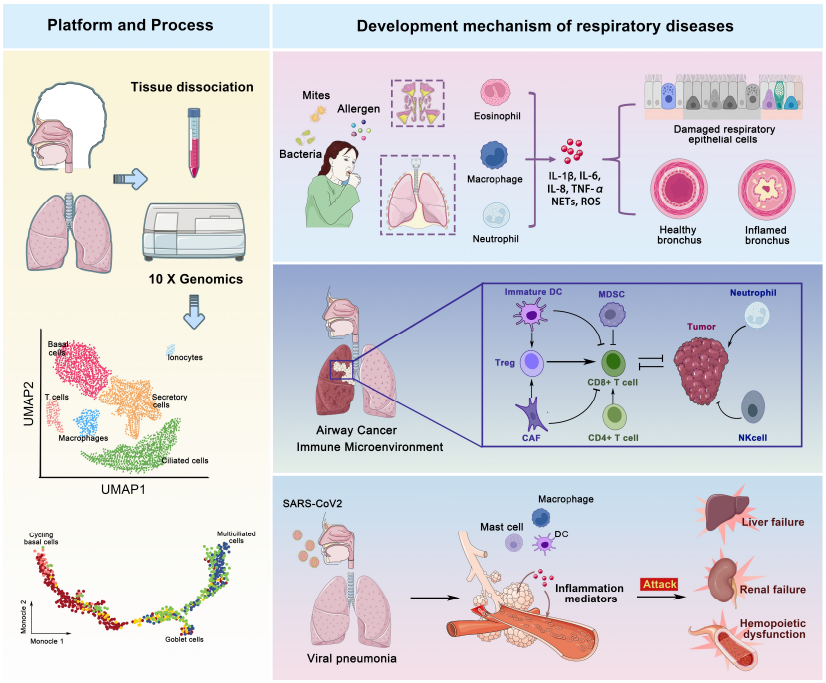

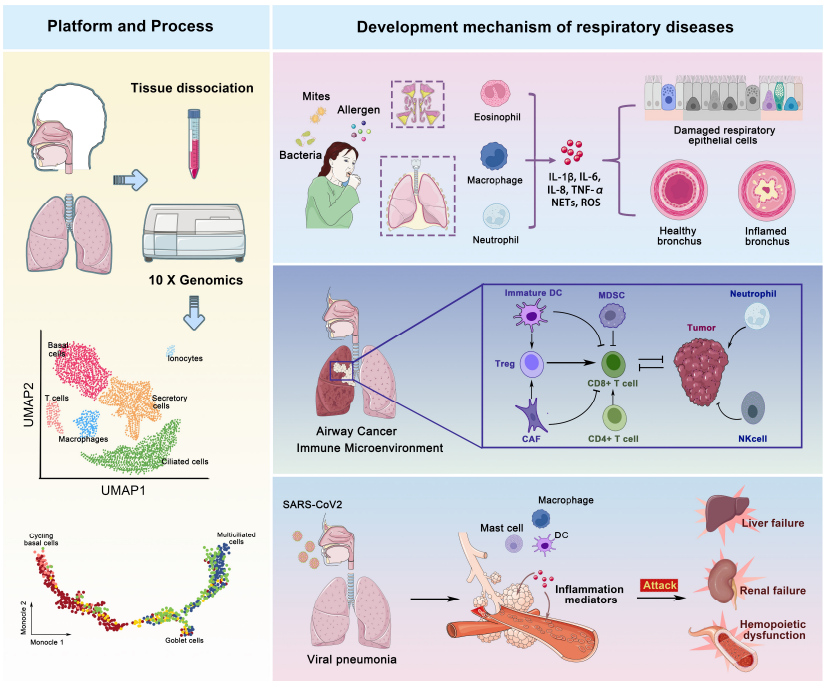

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