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REVIEW ARTICLE

Single-cell RNA Sequencing in Immunology

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Abstract: The complex immune system is involved in multiple pathological processes. Single-cell RNA sequencing (scRNA-seq) is able to analyze complex cell mixtures correct to a single cell and single molecule, thus is qualified to analyze immune reactions in several diseases. In recent years, scRNA-seq has been applied in many researching fields and has presented many innovative results. In this review, we intend to provide an overview of single-cell RNA sequencing applications in immunology and a prospect of future directions.

Keywords: Single-cell RNA sequencing, immunology, cancer, autoimmune disease, transplantation, tumors.

1. INTRODUCTION

The immune system is a combination of immune organs, immune cells and immune molecules, dominating multiple pathophysiology processes in organisms and keeping a physiology balance. However, due to the diversity of immune system composition and the complexity of immune reactions, exploration in immunology has been difficult, and the knowledge of it is restricted. Current exploring methods [1] based on the single experiment are inefficient and unstable, thus a simultaneous, high throughput method is urgently needed to detect whole anatomy of the immune system and the pathologic changes adapting to various diseases.

Single-cell sequencing (SCS) is a new technology to analyze genomics, transcriptomics and epigenomics on the level of single-cell, which complements the shortcomings of traditional high throughput sequencing by revealing homogeneity and heterogeneity among cells individually. As an emerging tool to analyze bioinformation, it is valued for its ability to collect cell information individually and identify all types of cells in a sample unbiasedly without prior knowledge for them, which would provide critical supports in understanding the diversity of the immune system.

In this review, we intend to offer an overview of the current methods and applications for single-cell RNA sequencing, displaying a general view of the capabilities of this technology in the immunology field.

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2. scRNA-SEQ: STATE-OF-THE-ART

The technology of single-cell measurement has constantly been improving in recent years. Yet steps of current single-cell technologies in the laboratory are still quite stable. A typical scRNA-seq protocol includes 7 steps [2]: single-cell isolation, lysis, reverse transcription, cDNA amplification, library preparation, sequencing and computational analysis. Among these steps, cell isolation, library construction and data analysis are paid most attention in single-cell RNA sequencing.

Cell isolation is the first step of scRNA-seq. Major cell isolation methods include limiting dilution, micromanipulation, flow-activated cell sorting (FACS), laser capture microdissection (LCM), microdroplets and microfluidics [3]. Current sequencing methods are composed of plate-based protocols, pooled approaches, and massively parallel approaches [4]. These methods isolate cells by microfluidics and microdroplets. Limiting dilution and micromanipulation is both ineffective for massive analysis. LCM is able to reserve spatial information, but the limited throughput and restricted sample form (tissue dissections) of this technology lead to a narrow application. FACS can separate cells with specific markers and filter out unviable cells before sequencing, therefore it is adopted in many experimental designs. However, to filter cells with certain known cell types subjectively before scRNA-seq could wipe out valuable hidden information in biosamples. Additionally, FACS does not really separate cells into individual analyzing units like a microplate or microdrop for further manipulation. Introducing an extra step or equipment will also introduce errors either.

Commonly used sequencing methods automatically manipulate cells to accommodate the handling of a massive

number of cells. Plate-based methods separate cells into different wells on a plate, whereas droplet-based methods capture cells in microdrops for further analysis. Both methods cannot ensure the accuracy of isolation [5]. Empty wells or microdrops may exist, and the inclusion of two (doublet) or more cells in a microwell or microdrop cannot be avoided. Such technical drawbacks introduce systematic error into the later analysis, in which every microunit is considered as a single cell. SMART-seq and SMART-seq2 are typical plate-based sequencing methods. The Fluidigm C1 platform, based on the SMART-seq2 method, has improved the manual separation process by using a microfluidics chip. Fluidigm also allows for visual monitoring of the wells during cell isolation to control empty or doublet wells. Droplet-based platforms, such as 10x Genomics, have high throughput separating ability. Despite some noise and loss with this batch manipulation method, the advantages of higher throughput and lower cost have made these platforms of choice. Of note, both types of platforms have pros and cons among isolation methods.

Library construction is performed to amplify the weak signals of mRNA in a single cell through reverse transcription and cDNA amplification. This amplification of the cDNA is performed by polymerase chain reaction (PCR), *in vitro* transcription (IVT) or SPLiT-seq [6, 7]. Although various protocols have been proposed to improve their efficiency and accuracy, none of these methods is perfect for all sequencing requirements. These methods can be classified according to the coverage of transcripts. Full-length transcript sequencing methods, such as Smart-seq2, SUPeR-seq and MATQ-seq, enable the detection of rare transcripts. SMART-seq2 can detect more genes within the same sample than other sequencing methods [8]. Although better sequencing depth is achieved by full-length sequencing, the inefficient procedures restrict the cell throughput. In comparing different groups of cells, the number of sequenced cells becomes a critical indicator. The high throughput and low cost of non-full-length sequencing methods, for example, 5′ sequencing methods, including Drop-Seq and 3′ sequencing methods such as STRT-seq, is an advantage in such cases. To perform massive and efficient analysis, all cDNA should converge in a pool. To avoid losing information on cell origin during this process, mRNA in the same well or droplet (not necessarily the same cell) is labeled with a barcode. In certain methods, unique molecular identifiers (UMIs) are designed to mark every mRNA before amplification, thus, enabling quantification of different transcripts from the same gene [9, 10].

Data processing requires bioinformatics analysis skills. As scRNA-seq retrieves enormous amounts of information, computational analysis is an important tool for drawing significant conclusions. After quality control, the cDNA library can be sequenced, thus, producing read data. Through barcodes and UMIs, information on the cellular and molecular origins of the read data is restored [5]. Further analyses are based on these restored data.

Library construction multiplies minimal biosignals, thereby allowing for the detection of mRNA in single cells but enhancing the data noise. Inviable cells, doublets (two cells in one droplet) and environmental RNA frequently contaminate the raw data. Quality control of the data is necessary for downstream analysis. Although general rules [11] and several analysis tools [5] exist for removing noise, determining the quality control strategy in a specific experiment still requires knowledge and experiments. Subjective factors among researchers may be involved during this process [5]. Reverse transcription also tends to yield biased results because of several factors, including gene length and sequencing depth. To correct the effects due to reverse transcription, normalization methods are used. A given normalization method cannot fit all types of sequencing data. The scone tool, apart from deducing the best normalization method according to data parameters [12], aids in deciding on a normalization method. After the trimming of raw data, subsequent analyses such as dimensional reduction and unsupervised clustering can be performed. To date, several calculation tools have been proposed, yet a consensus regarding the existing methods is lacking. Technical noise is not removed effectively, and the variability in cellular expression is not measured accurately [13]. The analysis of scRNA-seq data remains challenging.

scRNA-seq platforms are combined through different isolation and sequencing methods. Fluidigm C1 uses microfluidics and plate-based sequencing. 10x Genomics uses microdroplets and massively parallel approaches. BD Rhapsody uses microwell and massive parallel approaches and can be customized. The advent of commercial platforms has saved laboratory time and sequencing costs, thus allowing more laboratories to use scRNA-seq. Critical technological improvements played an important role in the commercialization of single-cell RNA sequencing. For example, Fluidigm C1, based on a switching mechanism at the 5′ end of the RNA transcript (Smart-seq), was developed in 2012 [14] and improved upon in the subsequent year [15]. Full-length sequencing of the transcript enables the detection of selective transcription isomers and single nucleotide polymorphisms. The improved sequencing coverage ensures less data loss. However, the high cost and restricted number of cells have prevented its extensive application.

Late in the subsequent year, the microdroplet method [16] became available, thus solving the problem of high cost and low throughput. With microfluidics technology, microdroplets of water in oil are generated. Barcodes are trapped in these structures together with single cells by using specific markers for the transcriptomes of every single cell. Subsequently, the cells can be mixed and lysed without the confusion of cell identity, and cDNA libraries can be generated. Given a large number of cells, cluster analysis or pseudo time analysis can provide further information on subgroups or developmental trajectories. Despite increasing sequencing information and decreasing costs, droplet-based methods cannot measure the full length of the target sequence [17], thus resulting in a loss of sequencing depth. Currently, researchers use both methods in a supplementary manner. DropSeq methods can be used to investigate the full range of cells, among which those of interest are selected for sequence and by SMART-seq2, covering both coverage and depth of sequencing.

Immune repertoire sequencing is a promising tool to determine the status of the immune system. Comprising the sum of T cell receptors (TCRs) and B cell receptors, the

Table 1. Comparison of different scRNA-seq platforms.

Platform	Cell Isolation	Library Construction	Capturing Efficiency	Cost ($ Per Cell)	Cells Sequenced
Fluidigm C1	Microfluidic	Full-length sequencing [70]	10% [70]	3.5 [6]	96 or 800 [70]
BD Rhapsody	Microwell	Cell barcode + UMI PCR RT and template switch [71]	79% [71]	3-6 [71]	100 – 10,000 [71]
10X Genomics	Microdrop	Cell barcode + UMI PCR RT and template switch [72]	65% [6]	0.5 [72]	1,000-80,000 [6]
indrop	Microdrop	Cell barcode + UMI IVT RT + 2nd strand synthesis [72]	7% [9]	0.25 [72]	7,000 [9]
dropseq	Microdrop	Cell barcode + UMI PCR RT and template switch [72]	5% [6]	0.1 [72]	5,000 – 10,000 [72]
Wafergen ICELL8	Microwell	Cell barcode + UMI PCR RT and template switch [73]	30% [74]	3 [74]	500 - 1,000 [73]
Illumina/Bio-Rad	Microdrop	Cell barcode IVT RT + 2nd strand synthesis [75]	3% [74]	1-4 [6, 74]	500 - 10,000 [6, 74]

RT, reverse transcription; UMI, unique molecular identifier; IVT, *in vitro* transcription.

immune repertoire reflects the pathological state of the organism. As the heterogeneity of TCRs/B cell receptors originates from the random transcription and combination of multiple noncontiguous gene segments (variable, diversity and joining, or V(D)J), genomic DNA and mRNA, despite having different advantages, can both be used for library construction. The invention of next-generation sequencing enabled massive immune repertoire sequencing, yet these methods sequence mixed nucleotides from different cells in bulk samples. Although these mixed nucleotides can provide general information on V(D)J recombination, they cannot reveal the pairing states of alpha/beta chains (for antigen-specific αβT cells) or light/heavy chains (for B cells) in single cells [18]. Therefore, the biological functions of these lymphocytes remain undetected. Single-cell sequencing with barcodes for identifying each cell appears to be an ideal tool for retrieving this critical information, despite decreasing throughput for searching for rare V(D)J recombination. However, single-cell sequencing of the immune repertoire is not a simple and comprehensive solution. Sequencing platforms must achieve a balance among throughput, read length and error rate. Raw data must be corrected with methods such as UMI to meet the accuracy needed at the single-cell and nucleotide level. RNA sequencing can reliably distinguish among cells and provide possible evolution maps. However, the expression of RNA and protein may differ, and proteomics should not be ignored in investigating humoral immunity [19]. A combination of immune repertoire, antibody proteomics and cell transcriptome analyses should provide a comprehensive portrait of the immune system.

In recent years, scRNA-seq has developed rapidly. Transcriptome *in vivo* analysis (TIVA) was introduced by Lovatt *et al.* [20] to include spatial information for single cells and account for the cell microenvironment. Guided by a cell-penetrating peptide, a TIVA tag is transported into living cells. Then, by selective laser-mediated photoactivation, TIVA tags are activated and combine with the mRNAs in the cell. TIVA tags and mRNAs are retrieved for further interrogation. Despite preserving accurate spatial information with a laser, this method is apparently insufficient for massive cell analysis. Upstream of transcriptomics, epigenetics is also included in single-cell sequencing studies. Assay for Transposase Accessible Chromatin with high-throughput sequencing (ATAC-seq) [21] was invented in 2013 and has rapidly developed since then. This innovative epigenetic technology can test for chromatin accessibility with Tn5 transposase. The transposase, tagged with known barcodes, binds accessible regions on chromatin. The barcodes are then sequenced and detected to confirm the accessible regions. ATAC-seq technology, compared with previous methods, including DNase-seq and FAIRE-seq, provides greater convenience and data validity in epigenetics studies [21]. With its higher sensitivity, ATAC-seq can detect epigenomics at the single-cell level. Single-cell ATAC-seq was invented later in 2015 [22] and was commercialized on the 10x Genomics platform in 2018. It is an important complement to scRNA sequencing upstream of transcription.

Innovations in measurement tools have provided powerful motivation for scientific studies. With the ability to detect higher dimensional information, combined temporal and spatial information and epigenetics information for individual cells, investigating many aspects of immunology has become accessible. Current knowledge of the immune system is rapidly increasing (Table **1**).

3. THE APPLICATION OF scRNA-SEQ

ScRNA-seq has superior abilities to those of traditional immunology methods in many fields. The advantages of scRNA-seq are based on its greater sensitivity than that of most traditional methods and its ability for detection without prior knowledge. Reviewing the related literature, we found that single-cell sequencing is currently mainly used in the following aspects (Table **2**).

3.1. Cell Anatomy: Searching for Rare Subtypes

Every organ consists of various cell types. Here, we take the kidney as an example. The kidney tends to be affected by multiple immune diseases, such as IgA nephropathy and lupus nephritis. However, understanding the immune conditions of the kidney remains limited. The complex immune composition and immune cell distribution have impeded further exploration of the kidney. In disease, specific cells and molecules may potentially serve as biomarkers for diagnosis or even provide new treatment opportunities. In 2018, the first scRNA-seq study on the kidney was conducted. Park *et al.* [23] found 21 cell types in mouse kidneys, three of which were newly defined. The comprehensive kidney cell atlas contains both kidney cells and immune cells. Diversity of immune cells exists in the kidney, including macrophages, neutrophils, B and T lymphocytes, and NK cells. Kidney cells are usually classified according to features such as physiological functions and anatomical positions [24], omitting information on transcriptomics. ScRNA-seq provides transcriptomic information that can link specific cell types to genetic diseases and distinguish new cell types.

Dendritic cells (DCs) in the human body have an important role in presenting antigens. Apart from plasmacytoid dendritic cells (pDCs), two subsets of conventional DCs have unknown origins and differentiation pathways. Using massively parallel single-cell mRNA sequencing and combining complementary bioinformatic approaches, See, *et al.* [25] deduced a group of pre-DC cells able to differentiate into cDC1 and cDC2; they then confirmed the results by using other algorithms. Interrogated with CyTOF, these cells were found to be $CD123^+$, $CD33^+$ and $CD45RA^+$. These cells have been found to share many common markers with pDCs; thus, some previous conclusions about pDCs should be revised on the basis of these identifications [26-28]. Pre-DC cells had been discovered 2 years before this research [29]. A much larger population of this pre-DC cell group by scRNA-seq has been found, thus, indicating the sensitivity of this method. However, scRNA-seq alone was not sufficient to confirm a new cell type. CyTOF and cell functional studies were performed to validate this classification. Unique markers of $CX3CR1^+CD33^+$ and the unique abilities of polarization, inducing proliferation of naïve CD4 T cells and production of IL-2, were confirmed in this cell group, thereby distinguishing pre-DCs from conventional pDCs.

In 2018, Crinier *et al.* [30] sequenced NK cells in the peripheral blood and spleen of mice. Although both groups of cells are denoted NK cells, their transcriptomes showed such different patterns that they could not be separated into the same cluster through unbiased analysis. Spleen and blood NK cells in humans and mice were divided into 4 and 2, and 3 and 2 groups, respectively, in an unbiased manner. In addition, according to the functions of the genes highly expressed in spleen NK cells, these cells might be more active than their relatives in peripheral blood. Although differences were found, two groups of NK cells were shared by both organs and both species, and were defined as NK1 (CD56dimCD27lo) and NK2 (XCL1) cells. The results indicated that some immune cells are almost specifically tissue-resident instead of being present in the lymph system [31]. Immunologists are now paying greater attention to tissue-resident immune cells. The proportion of these immune cells is so small in whole organs that few methods are sufficiently sensitive to investigate them. Notably, the scRNA-seq method has not identified conventional $CD27^+CD11b^+$ double-positive cells, which were previously found to be a transient maturation stage of NK cells. The authors questioned the existence of double-positive cells; dropouts in scRNA-seq should be considered a possibility.

The accuracy and sensitivity of this technology are indispensable in exploring unknown cells, yet proper sequencing

Table 2. Comparison of scRNA-seq to traditional experimental methods in immunology applications.

Applications	Advantages of scRNA-seq	Disadvantages of scRNA-seq
Cell anatomy	Detailed information on transcriptomics Providing with new classifications	Data analyzing tools are insufficient Requiring further validation Dropouts in a single measurement introduce uncertainty
Cellular markers	Providing with new markers Unbiased confirmation for conventional markers	Transcriptome is not a direct evidence Requiring further validation
Evolutionary relationships	Studied within one timepoint	Data analyzing tools are insufficient Hard to distinguish between the concurrent phenomenon
Comparison between immune status	Sensitive to rare cells	Requiring further validation Data analyzing tools are insufficient
Translation between species	Comparing cells of different species unbiasedly	Sensitive to different manipulating methods

strategies should be chosen to balance sequencing depth and sample amounts. Extensive efforts are necessary to distinguish false-positive results in rare data.

3.2. Redefining Cellular Markers

Cell markers are defined on the basis of either occasional findings or existing knowledge. In addition, methods to explore new markers are not unified or standardized. Thus, these markers are not regarded as comprehensive or essential. With scRNA-seq, markers of both known and unknown cells can be assessed in an unbiased manner.

Cell development in the mammalian lung remained poorly understood for years, partly because of the lack of lineage markers and the confounding effects of traditional sequencing methods [32, 33]. Sequencing the mouse lung epithelium late in sacculation, Treutlein *et al.* [34] separated these cells into five subgroups based on single-cell transcriptomes. Except for four known subtypes of cells, another subgroup showed gene expression intermediate between that in AT1 and AT2 cells, thus indicating a transitional subgroup between them or a bipotential precursor. Further interrogation of younger mouse lung samples indicated that the group was a precursor. In addition, the numbers of new mouse lung linage markers were distinguished by unbiased data analysis, which could potentially be more specific than the original markers. These enriched markers also indicated cell functions, illustrating a broader view of lung lineage cells. The full life cycle of Sftpc1 cells was traced, and seven gene sets of robustly multipotential, bipotential, nascent and mature AT2 cell states were identified, thus indicating the promise of this strategy for characterizing developing and mature cells. However, because full coverage of the target transcriptome is difficult to attain, and false replications during library construction are inevitable, scRNA-seq data are always one step away from the truth, as with all measurement methods. Reliable methods, such as CyTOF, for defining cell types should additionally be used to enhance scientific conclusions [25]. Specifically, in Treutlein's research, the clustering was confirmed by known markers. Other innovative findings have been based on this clustering [34].

3.3. Discovering Evolutionary Relationships

Cells in a microenvironment are complex to investigate immunologically. Cells with different characteristics can be distinguished, and similar cells can be clustered, perhaps in a developmental sequence. The development of cells is a continuous process. Thus, by sequencing all states of cells in a sample, changes in the composition, structure and function of cells during development can be revealed. This analysis method is powerful for interrogating the origins of stem cells and immune cells.

The fetus was believed to be protected from foreign antigens; however, certain types ($CD45RO^+$) of T cells are mainly found in the fetal intestine instead of mesenteric lymph nodes, fetal thymus or fetal spleen [35]. Work by Li *et al.* [36] in 2019, on the basis of the substantial overlap observed in CDR3 repertoires, demonstrated that foreign antigen exposure in fetal guts might explain this conflict. Using a pseudo time algorithm, Li *et al.* further interrogated a differentiation pathway for T cells according to a single cell expression profile. Transitions between cells could be deduced by the regulation of more than 1000 expressing genes, and three modules were distinguished. By considering the $CD4^+$ marker restricted by mass cytometry before scRNA-seq, the authors found that the expression profile of the 2nd module mainly concerned transcription, whereas cellular activation and immune regulation functions were found in the 3rd module, thus, indicating a development pathway of regulatory T (Treg) cells. Massive data have provided a foundation for developmental trajectory analysis; however, the current analysis techniques are insufficient. Trajectory inference methods are used to perform a variety of accuracy, scalability, stability and usability analyses based on different data sets [37]. Concurrent phenomena are difficult to separate and may be easily confused [38].

3.4. Comparison between Physiological and Pathological States

With more detailed interrogation of the mRNA expression status of single cells, scRNA-seq also enables the discovery of new biomarkers for differentiating among cells. In the comparison of physiological and pathological states, further validation based on such discoveries has the potential to aid in the development of clinical diagnostic or treatment tools.

HIV infections can lead to a series of immune disorders in the human body; among all complications, neurocognitive impairment is highly associated with immune activation in the central nervous system. Clinical investigation of such an inaccessible tissue would be unrealistic; however, Farhadian *et al.* [39] took advantage of the sensitivity of scRNA-seq to sequence cells within cerebrospinal fluid, and compared the results to those of homologous cells in the peripheral blood. A tiny sub-cluster of myeloid cells was distinguished among all the cells; these cells showed significant overlap in the expression of several genes with the expression in microglia from neurodegenerative disease mouse models, as also measured by scRNA-seq in previous studies [40, 41]. In the simultaneous analysis of cells from different diseases [42, 43], differences in multiple diseases can preclude conclusions from being drawn. Using scRNA-seq in a specific disease allows for certain genetic diseases to be linked to specific cell types [23]. A more accurate understanding of pathogenesis can be developed on the basis of scRNA-seq. Analysis comparing cell subpopulations on the basis of transcriptomics alone can be difficult and may yield unpersuasive results [5].

3.5. Translating Experiment Results between Species

The unique cell identification method of scRNA-seq makes this method suitable for investigating differences in cell composition and the expression profiles between species through the comparison of transcriptomes between cells. This capability will be critical in selecting suitable experimental animals and extending the results of animal experiments to human studies.

Rodents are the most investigated animals except for humans. As readily available and quickly reproducing animals, they have been considered ideal models for many physiology and pathology states for hundreds of years. How-

ever, mice and rats are essentially different species from humans, and translation of results from the laboratory to the clinic has long been a problem. Similarities in transcriptomes could provide a well-defined standard to identify cells across animal species, thus potentially providing a translational opportunity for immunologists. NK cells in both humans and mice were analyzed in 2018 with scRNA-seq, and the NK cell anatomy in both species was identified and compared. Differences and similarities were observed between species, but the NK cells of both species shared a close functional pattern, as assessed by single-cell transcriptomes. Such comparisons could enable translation between human and mouse studies. Similar anatomical results in mice were obtained in 2017, when Halpern *et al.* [44] examined mouse liver with scRNA-seq and smFISH, and reconstructed relative spatial information on mouse liver cells. Three clusters of cells were detected in mouse liver. In the next year, Macparland *et al.* [45] extended the previous research by sequencing the human liver from a deceased donor. In this study, 20 groups of cells were identified by unbiased data analysis [45]; the authors attributed this difference to species discrepancy, dissociation methods, and dead or low viability cells. A controlled experiment in human and mouse livers might better explain these differences. Nonetheless, these experiments remain an inspiration for the applications of scRNA-seq.

4. scRNA-SEQ IN CANCER IMMUNITY

Dysfunction of the immune system is important in tumorigenesis, tumor development and metastasis. With single-cell sequencing, the compositions and roles of both the innate and adaptive immune cells in various tumors have been clearly revealed.

Because tumors are complex tissues, measurements based on average numbers leave out much information on rare cells. ScRNA-seq might provide a solution for this information loss. Cancer can be roughly divided into solid tumors and hematological tumors. Immune cells in the tumor microenvironment and the composition of hematological tumors are of concern in immunology.

4.1. Solid Tumors

The resident immune cells in pancreatic ductal adenocarcinoma (PDAC) are highly heterogeneous. To unravel this complexity, Peng *et al.* [46] have analyzed PDAC with scRNA-seq. A total of 5, 6, 5 and 8 sub-clusters were found for T cells, B cells, macrophages and fibroblasts, respectively, in PDAC, in scRNA-seq data analysis. Further investigation of the two main types of immune cells, T cells and macrophages, revealed that cell cycle-related genes, such as centromeric protein-A (CENPA) and centromeric protein-E (CENPE), were preferentially expressed in certain cell subtypes, thus indicating proliferation ability among these cells. Homing markers, such as chemokine receptor 7 (CCR7) and selectin of lymph cell (SELL), provided evidence of the immaturity of a group of naïve CD4 T cells. Additionally, a pseudo time analysis was performed, showing a transition from naïve T cell and memory T cells to Treg cells. Composition, expression and developmental trajectory analyses are typically performed in scRNA-seq. These analyses offer fundamental information on the immune status of diseases.

The T cells resident in tumor tissues is mostly dysfunctional. Restarting the intrinsic effectivity of dysfunctional T cells resident in tumor tissue is a developing research field. Despite being a critical precondition of immune therapies, whether the TCRs of these T cells are potentially effective remains unknown [47]. In 2019, Scheper *et al.* [47] isolated TCR α/β genes from tumor resident dysfunctional T cells, performed scRNA-seq, and transfected the cells into functional donor T cells to observe the potential of these TCRs. The authors then tested the TCRs in ovarian tumors. The results indicated that TCRs could not recognize tumor antigens (19/20 incapable), owing to a lack of antigens in the microenvironment. Similar results were observed in patients with colorectal cancer, thus indicating that TCR dysfunction may be a more extensive phenomenon than previously recognized. Single-cell immune repertoire sequencing may be an applicable tool in further studies.

In 2017, Zheng *et al.* [48] identified 11 T cell subgroups in tumor tissue, normal tissue adjacent to tumor and peripheral blood of six patients with hepatocellular carcinoma (HCC), by using simultaneous single-cell transcriptomics and TCR sequencing. Apart from a marked gene in $CD8^+$ T cells found to be responsible for the down-regulation of interferon-γ and poor prognosis, the data showed connections among T cells and their potential development trajectories. The results were in accordance with those from several previous studies [49, 50], and were more accurate and less biased than previous results. Treg cells infiltrating in HCC were found to be recruited from the periphery, because they were unique, whereas exhausted $CD8^+$ T cells were more likely to be a result of local expansion. These results deepened the understanding of tumor-infiltrated T cells and might be important for immune therapies for HCC in the future. In addition, scRNA-seq demonstrated a unique ability to facilitate the exploration of tumor-related T cells, despite the complicated microenvironments and cell compositions in tumors.

Tumor-infiltrating lymphocytes are also important targets for diagnosis and treatment. For example, tumor-infiltrating myeloid (TIM) cells are promising immunotherapy targets in the tumor microenvironment; however, the subgroups and markers of these cells are unknown, and the indicators are sparse in simultaneous interrogation of groups of TIMs. Zilionis *et al.* [51] sequenced this complex group of cells by using the inDrop method in both human and mouse lung cancer samples. Although TIMs showed several similarities between human and mice, myeloid cells in the peripheral blood and those infiltrating tumor sites showed marked discrepancies, thus indicating the limitation of translational studies. Moreover, although the patients' tumors had different cell states, TIMs in their microenvironments overlapped well, thus indicating the potential for research on the essence of tumor immunity. Some specific genes within the TIMs were clearly associated with patient survival. These results indicated a potential field of research in translational medicine that could be associated with the essence of tumor immunity.

In 2018, Zhang *et al.* [52] developed an integrated method named single T cell analysis by RNA sequencing and

TCR tracking (STARTRAC) to examine the developmental trajectory of tumor-infiltrating lymphocytes. Based on single-cell transcriptomics and TCR sequences, STARTRAC demonstrated that in colorectal cancer, $CD8^+$ effector and "exhausted" T cells originate from tumor-resident T memory effector cells, and identified two IFNγ+ Th1-like cell clusters associated with various potential therapeutic effects [53] and immune checkpoint inhibition. In the same year, in non-small-cell lung cancer, Guo *et al.* [54], by using similar methods, found two groups of "pre-exhausted" $CD8^+$ T cells associated with better prognosis in lung adenocarcinoma, thus validating the TCR-based T cell tracing method.

4.2. Hematological Tumors

Beyond analysis of the composition of typical solid organ tumors, hematopoietic tumors can also be deeply analyzed by scRNA-seq. Hematopoietic tumors primarily originate from mutated or pathological immune cells. Current scRNA-seq methods have not achieved the sensitivity necessary to detect gene mutations among these immune cells. In 2017, Giustacchini *et al.* [55] proposed a sensitive method to analyze the same single cells by combining FACS, high sensitivity mutation detection and whole-transcriptome analysis. With this method, the researchers interrogated chronic myeloid leukemia (CML) stem cells from patients and distinguished a subgroup responsible for drug resistance, thereby partly explaining the sparsity of complete elimination. A comparison between CML stem cells and normal stem cells from the patients also indicated the possibility of CML related cell-extrinsic disruption. The authors believe that the same sequencing method could be adapted to similar malignant diseases to provide new insights.

Bulk analyses of chronic lymphoid leukemia (CLL) have revealed possible gene mutations, yet focusing on only the genome would sufficiently reveal the figures of CLL [56]. To determine the correlation between the genome and transcriptome, Wang *et al.* [57] used a two-stage RT-PCR amplification strategy with scRNA-seq and found that two genes, LCP1 and WNK1, are responsible for convergent evolution in CLL, thus, causing cells with different mutated genes to show similar expression states. Notably, scRNA-seq (by Fluidigm C1) indicated the transcriptional states and enabled general comparison between cell groups, but the gene coverage of the data is a weakness. The researchers reported that many dropouts occurred, and targeted qPCR was used to compensate for the sequencing data.

Whereas Fluidigm C1 cannot accurately detect single nucleotide variants (SNVs) [57], the scRNA-seq method described by Zheng *et al.* [58] has an impressive ability in sequencing SNVs. On average, as many as 350 SNVs were detected in every cell; this number was so high that the method was able to identify cell origins according to the SNV patterns among many types of immune cells after bone marrow transplantation. A possible population of the atypical blast and granulocyte precursors relevant to AML chemical therapy resistance has been found through this method [58]. An extremely high level of blast cells and immature erythroid marked by $CD34^+$ and $GATA1^+$ was being found to be associated with disease relapse in AML and with poorer prognosis.

ScRNA-seq is sufficient to depict and compare general transcriptomes in hematological tumors, and to search for possible reasons for drug resistance or pathological features. However, it is not an answer to everything. Drawbacks exist, although improvements are being made. Both these aspects should be considered when scRNA-seq is used.

5. scRNA-SEQ IN AUTOIMMUNE DISEASES

Autoimmune diseases cause tissue damage due to the body's immune response to their own antigens. The primary cause is the impairment of immune homeostasis, and multiple congenital and acquired factors are involved in the pathogenesis. However, the clinical course of autoimmune diseases greatly varies among different pathologies and individuals. The pathogenesis of autoimmune diseases remains unknown. This heterogeneity hinders immunologists' deeper understanding of autoimmune diseases.

The etiology and pathology of rheumatoid arthritis (RA) are unknown. Apart from clinical symptoms, pathological thickening of the synovial lining composed of lymphocytes, macrophages and dendritic cells amid the original subintimal synovial fibroblasts is a critical feature of RA. In 2018, Stephenson *et al.* [59] deployed a 3D-printed low-cost droplet-based sequencing to the synovial tissue from five patients with RA. Fibroblasts were primarily analyzed for their critical influence on fibrous tissue formation. Meanwhile, typical immune cells in fibrous tissue, including $CD4^+$, B and NK cells, were identified and analyzed. One subgroup of NK cells expressed XCL1 and XCL2, chemokines associated with fibroblast secretion. The authors suggest that the results provide the first atlas of hematopoietic and fibroblast cells in autoimmune diseases [59]. The invention of a portable sequencing device also made this method practical in clinic settings. In the same year, Kernfeld *et al.* [60] first took advantage of a combination of single-cell sequencing and machine learning to analyze the whole thymus. Some rare non-conventional lymph cells were first discovered. The susceptibility to autoimmune diseases is generally accepted to be associated with genetics and developmental defects. As verified in this research, BLD4 (myeloid) and BLD5 (non-conventional lymph cells) acquired from an autoimmune patient showed significant up-regulation of autoimmune-related genes. These single-cell analyses of autoimmune diseases display a capability to reveal cell pathology and the nature of diseases, thus providing valuable information for future studies. Advanced sequencing tools and algorithms were applied in this analysis.

Single-cell sequencing can also help scientists identify new targets for diagnosis and treatment. Immune biotherapies have not always been effective in ileal Crohn`s disease (CD). According to therapeutic effects, the patients can be divided into two groups; however, pathological differences between these two groups had not been found, owing to limited biomarkers and knowledge of the disease model of CD. With scRNA-seq, Martin *et al.* [61] searched the cellular landscapes in CD and found a GIMATS module, identifying failure to achieve durable CS-free remission after anti-TNF therapy in a pediatric inception cohort. Further research was performed using bulk transcription in four independent CD cohorts, including 441 patients, and all samples verified the

validity of this module. The module could be used in clinical diagnosis to distinguish patients resistant to certain therapies and achieve more precise treatment, because the GIMATS module can be detected without expensive scRNA-seq. Similarly, Der *et al.* [62] have analyzed skin and kidney biopsy samples from patients with SLE with Fluidigm C1, and detected a type I interferon signature in both skin and kidney biopsy samples from patients with active SLE, thus, indicating a potential lupus nephritis diagnosis method by skin biopsy. The discovery of new markers is promising for disease diagnosis, and scRNA-seq is specialized in finding novel cells and molecules. These studies indicated a strong potential of scRNA-seq in examining diseases with unknown pathology. After the most critical problem is conquered for a disease, diagnosis and accurate treatment would be expected to naturally emerge.

In autoimmune diseases, scRNA-seq is broadly adopted to examine etiology, pathology, clinical diagnosis and treatment. The ability to explore the unknown has become a critical feature in this field. Because transcriptome sequencing does not depict the whole picture of a cell, future directions in proteomics and genomics should be complementarily used for a thorough investigation of autoimmune pathology.

6. scRNA-SEQ IN TRANSPLANT IMMUNITY

Transplantation has become a relatively mature therapy to rescue end-stage patients in the past 70 years, yet it still faces new problems [63]. During treatment, the immune system fiercely attacks extraneous organs. Although controlled by immunosuppressive drugs, the harmful effects remain fatal to the graft. In addition, immunosuppression therapy after transplantation results in several complications, thus, reducing the expected survival time of the recipient. Although few restricted transplant studies have used scRNA-seq, they have inspired other studies in the field.

Last year, Wu *et al.* [64] first attempted to perform scRNA-seq on human kidney samples. The comparison between a rejection case and a healthy human kidney showed a wide range of inflammatory responses. The authors also found a special non-classical group of monocytes expressing both typical monocyte markers and a panel of mature DC markers. A comparison of this group of monocytes and a peripheral blood mononuclear cell dataset showed a clear boundary between groups, thus, indicating a novel cell group that appears to be differentiating from monocytes to DCs. A reassessment of putative disease-related genes was also performed. The 30 genes indicating T cell-mediated rejection were expressed in mostly T cells, thus, providing an ideal theoretical basis for prediction based on these genes. Wu *et al.* [64] have also found that some genes previously believed to be responsible for endothelial cell (EC)-related allograft pathologies were not specifically expressed in ECs. Some were even specifically expressed in non-EC cells. However, transcripts enriched in antibody-mediated rejection coincided well with EC cells, thereby indicating a possible specific relationship. Early phage molecular markers could be identified by scRNA-seq through these trials. The 10x Genomics platform and InDrop methods were more suitable for analyzing these biopsy specimens than standard DropSeq approaches, possibly because of the incomplete cell lysis in DropSeq. Applying scRNA-seq to graft biopsies has been demonstrated to have potential, yet obstacles still remain. The major challenge is protecting RNA from degradation during dissociation.

The liver is critical in both metabolic and immune functions in the human body, yet its immune microenvironment is poorly understood. Using 10x Genomics scRNA-seq, an overview of the hepatic immune microenvironment can be illustrated. Macparland *et al.* [45] performed scRNA-seq on donor livers to generate a human liver cell atlas, thereby deepening understanding of the physiological environment and immune composition in healthy human livers. On most occasions, collecting liver samples from healthy individuals would be unethical; therefore, the researchers used the caudate lobe of a deceased donor's liver, which was abandoned before transplant surgery. This research offers new prospects for both clinical studies in transplantation and human cell atlases.

Studies in lung transplantation are also rapidly progressing. Pathological changes widely occur in cells in tissues with lung fibrosis [65]. Except for cells such as alveolar type I and type II cells, immune cells such as macrophages, NK cells, B cells, monocytes, DCs, plasma cells and mast cells showed an array of fibrosis-related gene expression changes. With droplet-based microfluidics, 17 clinical tissue samples were analyzed, including six donor lungs and six receptor lungs. A total of 13 cell populations were identified, and patterns of disease changes in all types of cells were observed, including in most major immune cells. Chronic lung allograft dysfunction (CLAD) is closely associated with the long term survival of patients receiving lung transplantation. Currently, CLAD is a clinical diagnosis based on several diagnostic standards [66]; however, as with many other clinical diagnoses, once diagnosed according to clinical symptoms, the disease is too advanced to be cured or prevented from worsening. In 2017, Weigt *et al.* [66] explored early diagnosis by taking advantage of scRNA-seq. CLAD samples (surveillance bronchoalveolar lavage samples) were retrieved from patients who had developed CLAD 2 years after sample collection. No differences were detected in the total cell number or cell distribution, whereas in CLAD samples, immune function associated genes in cytotoxic lymphocytes were activated, and the reactome and KEGG pathways of immune activation abilities were monitored in the CLAD samples. The researchers concluded that this sequencing method can distinguish clinical pre-CLAD and CLAD-free patients, thus, potentially improving the diagnosis and prognosis of lung transplant patients.

Bone marrow transplantation is the last hope for hematopoiesis tumor patients, yet immunosuppression treatment and chemical medication are difficult to balance. The ability to detect donor and host cells in immune cell chimerism is vital in hematopoietic stem cell transplantation to predict the success or failure of this therapy, yet current clinical methods remain to be improved. By mixing known cell line samples (Jurkat and 293T) with those from three healthy donors, Zheng *et al.* [58] tested the ability of two separation methods. Fresh PBMCs were also obtained from two patients with HSC transplantation and AML to further demonstrate the feasibility of the sorting method based on SNVs. Donor

and host cell proportions were accurately examined and confirmed by independent clinical chimerism assays. Compared with previous methods, this SNV-based detection method performed better in detecting donor and host cells closely matched in genotype, and detecting cells with unknown genotypes. This method may lead to better monitoring and patient survival.

After transplantation, although controlled by immunosuppressive drugs, harmful effects can be fatal to the graft. Detection of early subtle damage is necessary for graft receptors. Potential markers detected in these experiments could provide opportunities for life-saving diagnosis. ScRNA-seq acts as a sensitive diagnosis instrument in transplant immunity, thus leading to a better understanding of the immune state of transplant receptors.

CONCLUSION AND PROSPECTIVE

ScRNA-seq has become a powerful tool in analyzing immune bioinformation in recent years. Currently, it is a developing technology with many methods and platforms for researchers to choose from, each with unique pros and cons. New sequencing methods [67-69] are being developed and hold great potential.

The ability to retrieve massive information from individual cells, without preliminary knowledge of target cells, has enabled scRNA-seq to surpass other methods, such as FACS, bulk RNA sequencing and immunohistochemical staining. These characteristics are particularly suitable for examining complex cell compositions and microenvironments, such as tissue-resident immune cells, developmental biology, tumors and the tumor microenvironment, autoimmune pathology and immune cells in transplantation. Extensive progress has been made in these fields.

Apart from these achievements, advanced single-cell sequencing methods are emerging rapidly. Chromatin accessibility in single cells can now be sequenced by scATAC-seq, which enables accurate measurement of epigenomics by using hundreds of cells. Adaptive immune cells used to be extremely difficult to interrogate, yet with the development of immune repertoire sequencing, diversity among T cells has become easier to measure. The sequencing process of single cells requires dissociation of tissue samples, thereby disrupting the spatial information. To restore the spatial information for further investigation, researchers can take advantage of spatial transcriptomics methods by tagging each cell with RNA probes. Together these approaches have opened new gates leading to deeper biological understanding and clinical medicine, by increasing the data dimensionality and revealing more subtle and essential information.

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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