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Immunology in the Era of Single-Cell Technologies

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Abstract

Immune cells are characterized by diversity, specificity, plasticity, and adaptability—properties that enable them to contribute to homeostasis and respond specifically and dynamically to the many threats encountered by the body. Single-cell technologies, including the assessment of transcriptomics, genomics, and proteomics at the level of individual cells, are ideally suited to studying these properties of immune cells. In this review we discuss the benefits of adopting single-cell approaches in studying underappreciated qualities of immune cells and highlight examples where these technologies have been critical to advancing our understanding of the immune system in health and disease.

INTRODUCTION

The history of immunology is marked by significant breakthroughs in our understanding of the immune system: from the discovery of vaccination by Jenner in 1796 to the distinction between T and B cells by Miller and Mitchell in 1961–1968 to the discovery of immune checkpoint molecules by Allison and Honjo in the 1990s. These advancements in biological insight depend on the development of novel approaches and technologies. The invention of fluorescence-activated cell sorting (FACS) in 1979 allowed for the separation of distinct populations of cells based on expression of surface markers (1, 2), paving the way for the study of phenotype and function of immune cell types. The combination of microscopy with cell staining and development of genetically modified fluorescent reporter animal models in the 1970s (3) allowed for the visualization of T and B cell zonation in lymph nodes (4) and dynamic visualization of immune cells, including natural killer T cells and dendritic cells patrolling liver and intestinal tissues, respectively (5, 6).

Named method of the year in 2013 by *Nature Methods* (7), single-cell technologies are providing the possibility to interrogate the immune system at the level of individual cells. These approaches allow unprecedented resolution of immune cell phenotypes, communication networks, and plasticity, at varying levels of throughput and coverage. Moreover, these methods are increasingly adapted for unbiased assessment of cellular features, breaking away from the tradition of distinct cell type classification and allowing for reconstruction of their response dynamics. Combined with improvements in spatial techniques, single-cell methods are also helping to dissect immune cell-cell communication networks at the systems level.

In this review, we discuss the recent single-cell revolution in the context of immunology—with a focus on properties of immune cells and responses that have traditionally been difficult to study at the population level. Specifically, we cover how single-cell methods are helping to resolve cellular heterogeneity, spatial positioning within tissue, response and development trajectories, and T/B cell receptor repertoires, and we showcase recent studies that have adopted these strategies to make significant contributions to the field. Finally, we provide an outlook for single-cell methods in future research and clinical translation.

CELLULAR HETEROGENEITY

Heterogeneity refers to diversity within a population. In evolutionary terms, heterogeneity is advantageous—higher diversity within a population increases the chance that, following a significant environmental change, there will be some individuals with characteristics suitable for survival (8). The immune system similarly benefits from heterogeneity, with a diverse array of cell types and states needed to manage the array of pathogens. Heterogeneity exists at each level of molecule, from genome to phenotype, and ultimately, cellular function. At arguably the most extreme level of immune heterogeneity, T and B cells express a repertoire of T and B cell receptors (TCRs and BCRs, respectively) permitting their specific recognition and elimination of a range of foreign invaders (9).

Determining the full range of cells within the human body has traditionally been difficult, due to technical limitations restricting the number of parameters that can simultaneously be measured and the minimum amount of starting material required. Single-cell approaches overcome this restriction through their unbiased nature of assessment, and they are shedding light on previously unappreciated heterogeneity within immunity (**Figure 1**, **Table 1**). In this section we discuss the various single-cell technologies to measure cellular diversity on the genetic, epigenetic, transcriptomic, and proteomic levels.

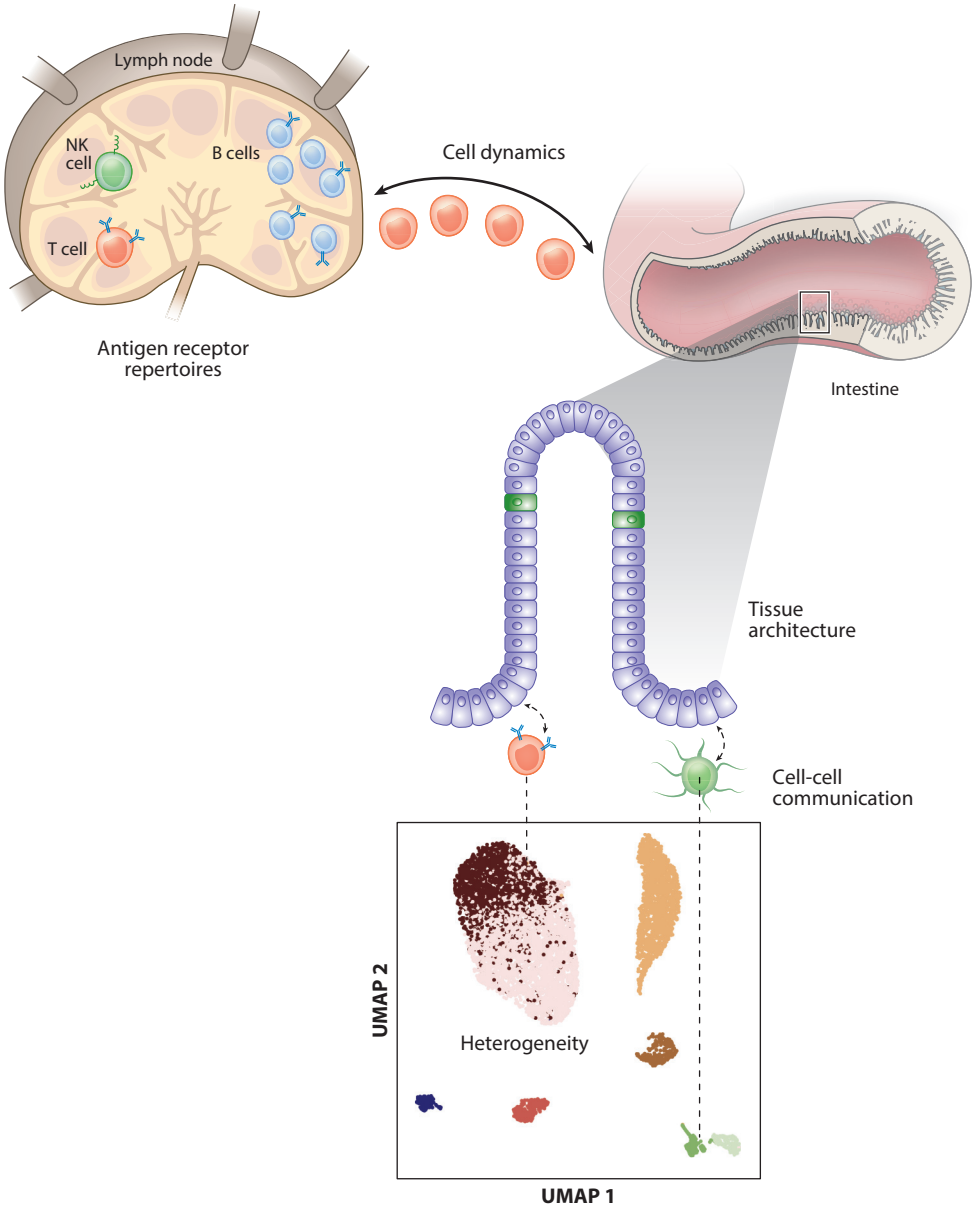


Figure 1

Schematic of properties of the immune system that can be studied through single-cell approaches. **Table 1** summarizes key examples from the literature. Abbreviation: NK, natural killer.

Heterogeneity Within the Genome and Genomic Regulation

As cells replicate, they can incur random mutations in their DNA, resulting in genomic heterogeneity. Most of these mutations have no consequence for cells, as they typically occur in nontranscribed DNA or result in the same or functionally equivalent proteins. Mutations in coding or promoter/stop regions, however, can cause loss of gene function or forced transcription.

Table 1 Summary of single-cell approaches and key examples from the literature

Immunological property	Key single-cell approach	Ref.
Antigen receptor repertoires	TCR/BCR/KIR sequencing paired with clonality analysis	14, 37, 50, 58, 107–118
Cell dynamics	Trajectory inference and lineage tracing	Reviewed in 48, 49; 61–67
Tissue architecture	In situ RNA profiling, in situ sequencing	138–151
Cell-cell communication	Ligand/receptor databases paired with single-cell sequencing	39, 123–127
Population heterogeneity	scWG-seq, scATAC-seq, scRNA-seq, CITE-seq, REAP-seq	12, 15–23, 46, 47, 210

Abbreviations: CITE-seq, cellular indexing of transcriptomes and epitopes by sequencing; REAP-seq, RNA expression and protein sequencing; scATAC-seq, single-cell assay for transposase-accessible chromatin using sequencing; scRNA-seq, single-cell RNA sequencing; scWG-seq, single-cell whole-genome sequencing.

Single-cell genome sequencing allows us to appreciate this genomic heterogeneity. In particular, single-cell genomics has been applied in oncology to understand the genetic heterogeneity within solid tumors and determine driver mutations underlying cancer progression and metastasis (10). Additionally, single-cell genomics has been applied to understand the genetic mosaicism of tissue. In a seminal study, Martincorena et al. (11) performed targeted single-cell genomics on healthy human esophageal epithelial cells. They were able to determine that by age 60 years, over 50% of epithelial cells had acquired cancer-associated mutations, and furthermore, the number one risk factor for the accumulation of these mutations was older age, rather than smoking or exposure to other common carcinogens. Tumor heterogeneity is discussed in more detail below.

Diversity in cell profiles may also be driven by heterogeneity in gene regulation. ATAC-seq (assay for transposase-accessible chromatin using sequencing) is a method for measuring open chromatin regions of DNA as a way of determining which genes are accessible for transcription. This method has been adapted for use at the single-cell level (scATAC-seq) in plates (12, 13), with microfluidics (14), and more recently with droplet-capture by 10× Genomics (Chromium Single Cell ATAC Solution), and it holds promise for understanding heterogeneity in the shift of immune cell states such as the formation of multiple effector or memory states.

Cellular Diversity at the Transcriptomics Level

The transcriptome is dynamic and tightly linked to cell identity and function. Single-cell methods that assess this modality allow for inherent features of transcription, such as transcriptional noise and regulation heterogeneity, to be measured. These methods typically measure eukaryotic polyadenylated mRNA and rely on dissociation of tissue to single-cell emulsions, followed by capture of individual cells in wells or droplets in which reverse transcription, barcoding, and downstream processing of complementary DNA (cDNA) libraries occurs.

Widely used single-cell RNA sequencing (scRNA-seq) protocols include Smart-seq and Smart-seq2 (15, 16), which apply template switching in combination with PCR to gain full-length reads, and cell expression by linear amplification and sequencing (CEL-seq) (17), CEL-seq2 (18), and massively parallel RNA single-cell sequencing (MARS-seq) (19), which use in vitro transcription. These methods tend to be lower throughput (i.e., hundreds of cells per experiments, with a potential to process thousands of cells across multiple runs), but often have deeper sequencing depth per cell.

Microfluidics-based approaches have enabled thousands to hundreds of thousands of cells to be processed in parallel. Droplet-based platforms such as Drop-seq (20) and InDrop (21, 22) facilitate assessment of large-scale cell numbers at low cost. Similarly, a nanowell-based approach, Seq-well (23, 24), can process up to 10,000 cells per array. 10× Genomics has commercialized

droplet-based methods firstly as their Chromium 3′ chemistry, where transcripts are captured on oligo-dT-tagged gel beads and transcribed from the 3′ end (23). In the Chromium Next GEM Single Cell 5′ Solution, the polydT sequence is substituted for a template switch oligo (TSO) allowing for sequencing of longer transcripts from the 5′ end.

The advances of single-cell transcriptomics technologies promoted development of innovative computational methods for analysis (for detailed best-practice recommendations please see 25). Briefly, the raw sequencing output from scRNA-seq can be fed into an analysis workflow that starts with alignment of the sequenced reads to a reference genome to produce matrices of molecular counts or read counts, depending on whether unique molecular identifiers (UMIs) are used in the single-cell library construction protocol. After the count matrix is produced, quality control metrics such as the number of counts, the number of detected genes, and the fraction of counts from mitochondrial genes per cell are applied in order to retain only cells with good integrity. Additional filtering of potential doublets (26, 27), cells affected by dissociation (28) or removal of background contamination of cell-free RNA, should also be considered. Downstream analysis such as normalization and dimensionality reduction algorithms to determine the variability within the cells are now part of standard packages for scRNA-seq analysis (29, 30). When combined with clustering and visualization algorithms such as *t*-distributed stochastic neighbor embedding (tSNE) or uniform manifold approximation and projection (UMAP), single-cell data can be used to identify the structure within cell populations, better profiling established cell types and identifying novel subtypes and states. In this way scRNA-seq has been implemented to understand subpopulations of myeloid cells (31–38) and lymphoid cells (38–42). Further analysis such as trajectory modeling, gene regulatory networks, and cell-cell communication networks inference enable deeper insights into cellular phenotype, dynamics, and function (**Figure 2**).

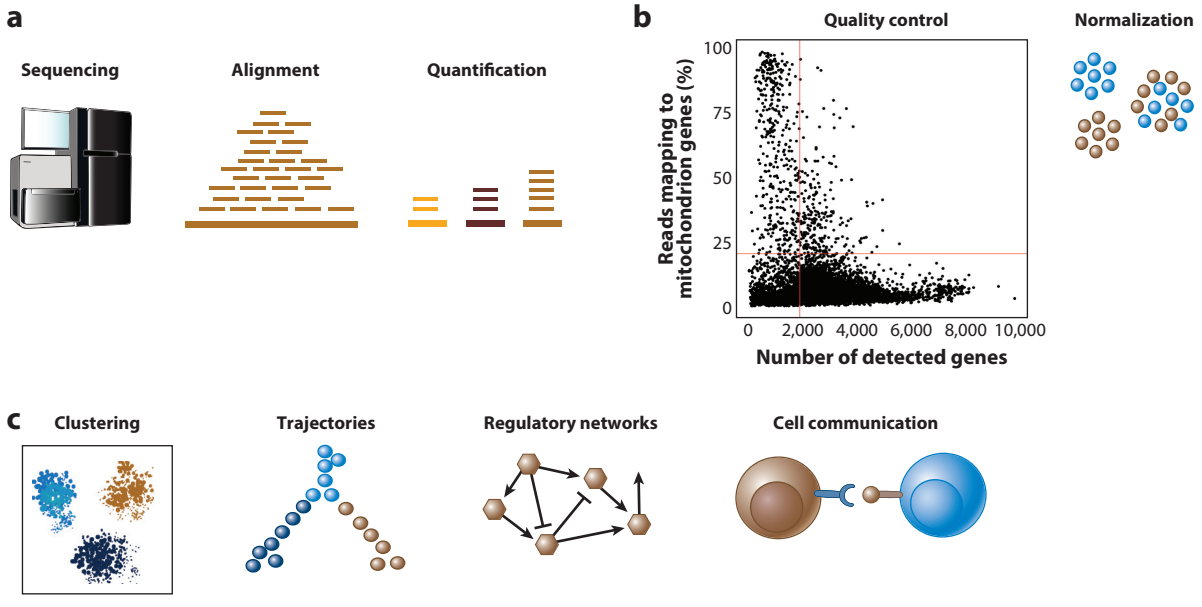


Figure 2

Schematic diagram of a standard single-cell RNA-seq data analysis workflow, including steps involved in (*a*) data acquisition, (*b*) data cleaning, and (*c*) data analysis.

Heterogeneity at the Protein Level

Proteins mediate cellular function; therefore, understanding heterogeneity at this level is particularly informative. Measurement of the entire proteome is limited by the inability to directly amplify proteins and due to the added complexity of secondary and tertiary structures. Current staples in immunology instead employ a targeted approach by conjugating either fluorescent tags (FACS) (43) or heavy metal isotopes (CyTOF) (44) to antibodies. While these techniques are limited in the number of parameters they can simultaneously assess—approximately 20 to over 30, respectively—they allow for assessment of 100,000 to millions of cells per sample.

Abseq is a method that uses conjugation of antibodies to unique DNA barcodes that are read out with microfluidic barcoding and DNA sequencing, and it has extended the number of proteins that can be assessed into the hundreds, limited mainly by the availability for high-fidelity antibodies (45). Recently, equivalent methods for use alongside scRNA-seq methods including Chromium 10× Genomics and Drop-seq have been developed, namely, cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) (46) and RNA expression and protein sequencing (REAP-seq) assay (47). In REAP-seq, antibodies against proteins of interest are covalently bonded to aminated DNA sequences, minimizing steric hindrance to allow the authors to demonstrate simultaneously assessment of 82 antibodies (47). CITE-seq, on the other hand, uses streptavidin-biotin conjugation of antibodies to unique DNA sequences and has been demonstrated with smaller antibody panels. Commercially available oligo-tagged antibodies compatible with both techniques are available as the Biolegend TotalSeq solution. Pairing of transcriptomics and proteomics achieved through these methods benefits from unbiased assessment of heterogeneity at the transcriptome level and additional diversity arising from posttranscriptional and posttranslational processes. These methods are currently restricted to assessment of cell surface markers, although they could theoretically be applied to quantify intracellular proteins including transcription factors. As an example of where these technologies would add value to understanding cellular heterogeneity in immunity, they can be used to measure splice variants, such as CD45RA and CD45RO, which would delineate naive from central memory T cells, currently nearly impossible with scRNA-seq alone. Additionally, joint clustering on both gene expression and cell surface proteins from the same cells could achieve much higher resolution in defining immune cell states (46).

CELLULAR DYNAMICS

Trajectory Inference

Immune cell heterogeneity cannot be entirely described by a discrete classification. Immune responses involve a complex network of diverse cell types, each of which can be at different stages in their differentiation or maturation. Furthermore, the phenotype of immune cells is highly plastic, shaped by their tissue and environmental context in steady state and under pathological conditions. To fully describe the identity of a cell, it is also important to trace its developmental origins and understand its lineage relationships with other cells. Therefore, reconstructing the dynamic landscape of the cellular identity is crucial to better understand immune responses both in homeostasis and disease, and to be able to therapeutically manipulate cell fate.

Developmental processes are driven by transcriptional changes that lead to cell differentiation and commitment to a specific lineage. Given the asynchronous nature of immunological processes such as hematopoiesis and differentiation, time series population-based data are not sufficient to model the dynamics of gene regulation. By capturing immune cells during a dynamic process, scRNA-seq generates static snapshots of the entire process that can then be modeled as a continuum of transitional cell states.

A plethora of computational approaches have been developed that enable building cellular trajectories from static snapshot data. Overall, these methods order cells on a trajectory according to their similarity in gene expression, assuming that the similarity is dominated by a specific dynamic process. Once a trajectory has been inferred, further analysis allows modelling branching behaviors to determine cell fate decisions and identify transcription factors that potentially control transitions from one cellular state to the next (for an extensive review see 48, 49). Single-cell transcriptomics combined with pseudotime inference algorithms has helped reveal key regulators of cell fate decisions during cell development and differentiation (50–53), molecular programs in cell migration and tissue adaptation (54, 55), and differentiation hierarchies in hematopoiesis (34, 51, 56). In addition, integrated analysis of developmental trajectories and immune repertoire has shown developmental relationships between cytotoxic and exhausted tumor-infiltrating T cells (57, 58), local expansion of T regulatory (Treg) cells in tumors (59), and potential for transdifferentiation between Treg cells and different T helper (Th) subsets (58).

Lineage Tracing

Trajectory inference approaches have provided a unique opportunity to track immune cells during differentiation and delineate lineage hierarchies. However, these are still descriptive trajectories that can only generate hypotheses. Resolving the mechanisms by which a single cell gives rise to different progeny requires direct tracking of cellular lineage and simultaneous measuring of the phenotype.

Lineage tracing through genetic labeling involves tracking of genetic features, either those implemented experimentally, through the introduction of exogenous material into cells (retroviral labeling, plasmid transfection, genetic recombinations, or CRISPR-Cas9 genome editing), or intrinsic features, by using naturally occurring variations inherited from one cell generation to the next (somatic mutations, copy number variations, or epigenetic markers) (60). In model systems, lineage tracing has been achieved using two strategies: imaging-based and sequencing approaches. Imaging-based methods retain the spatial positioning; however, they are limited in their temporal resolution. On the other hand, techniques that involve sequencing generally disrupt the spatial context of cells.

Recently, some of these approaches were integrated with scRNA-seq to obtain both cell lineage relationships and detailed phenotypic information at single-cell resolution. Kimmerling et al. (61) used off-chip scRNA-seq after multigenerational lineage tracking and staining of cells to look at both interclonal and intraclonal variability in activated $CD8^+$ T cells and showed that lineage-dependent transcriptional profiles correspond to functional phenotypes. Another recent technique called MEMOIR (memory by engineered mutagenesis with optical in situ readout) used barcoded recording elements whose state can be stochastically altered by CRISPR-Cas-based targeted mutagenesis and read out in situ by single-molecule fluorescent in situ hybridization (smFISH) (62). Genome editing approaches have also been combined with single-cell transcriptomics (63, 64). Alemany et al. (65) employed CRISPR/Cas9-induced genetic scars to study the clonal history of the hematopoietic system of zebrafish and showed that a small set of progenitors generate all hematopoietic cells in the kidney marrow.

The lineage tracing techniques that require genome editing to introduce genetic labels are constrained in their usability only to model organisms or in vitro systems. Somatic mutations in humans arise spontaneously over time, making it possible to use them as lineage markers for reconstruction of clonal relationships. However, naturally occurring somatic mutations generally have low frequencies, thereby requiring high-coverage sequencing. To overcome this limitation, two recent studies exploit somatic mitochondrial DNA (mtDNA) mutations as natural genetic

barcodes to reconstruct cell lineages and infer clonal dynamics in scATAC-seq and scRNA-seq data (66, 67). In addition, intrinsic T and B cell receptors can be used to trace clonality and lineage relationship for these lymphocytes, which is expanded upon in the next section.

The integration of single-cell lineage tracing and transcriptome profiling provides validation of the inferred trajectories and, at the same time, refinement of the lineage reconstruction and inference of the dynamic rates at which cells switch between states (68). Understanding lineage relationships between cells provides additional temporal resolution to study dynamic processes and can reveal insights into fundamental developmental processes as well as pathologies such as cancer. Ultimately, these approaches will be able to reconstruct not only individual lineages, but also complete tissues and organisms.

Regulatory Landscape of the Immune System

Gene expression is tightly regulated by networks of transcription factors and signaling molecules. Single-cell technologies offer an opportunity to study the underlying regulatory programs that define and maintain cellular identities and transcriptional states. The increasing sizes of single-cell data sets are improving statistical power and enabling inference of more complex dependencies between genes. In addition, the combined stochastic and regulated variability between cells provides inference of more accurate and context-specific networks.

scRNA-seq is the leading single-cell technology for studying gene regulation. Advances in single-cell transcriptomics also prompted the development of computational approaches for reverse-engineering gene regulatory networks from scRNA-seq data. One class of method has been adapted from bulk analyses and aims to identify direct regulatory interactions between transcription factors and their targets based on coexpression analysis (69, 70). Other methods combine inferred trajectories with coexpression analysis to build gene regulatory networks from dynamic processes (71–75) (for a more detailed review see 76, 77).

Although scRNA-seq allows for measuring cellular heterogeneity, regulatory processes are too complex to infer from the transcriptome alone. The epigenomic landscape shows which regulatory regions (promoters and enhancers) are crucial for each state and provides a complementary measurement of cellular identity. Recent advances in epigenomics include scATAC-seq (12, 78, 79), chromatin organization [Hi-C (80) and nuclear lamina interactions (81)], histone modifications (82), and single-cell DNA methylation profiling (83–86). However, due to the limited efficiency of current protocols and the signal at any genomic locus being constrained by DNA copy number, most of these methods provide sparse and noisy data that pose challenges for analysis and interpretation. This prompted development of novel computational methods that overcome these issues and provide an unbiased characterization of cell types and the regulators that define them (87–90). A number of recent studies have demonstrated the applicability of these approaches in studying the regulatory landscape of human hematopoiesis (91, 92), to identify shared regulatory programs driving $CD8^+$ T cell exhaustion and $CD4^+$ Tfh cell development in basal cell carcinoma patients treated with PD-1 blockade (93) and to reveal dynamic changes in chromatin accessibility in effector, memory, and exhausted $CD8^+$ T cells in response to in vivo viral infection (94).

Emerging technologies now provide combined measurement of multi-omics data from the same cells. These include parallel extraction of the transcriptome and methylome [M&T-seq (95), sci-CAR (96)]; transcriptome, accessible chromatin sites, and DNA methylation [scNMT-seq (97)]; and transcriptome and proteome (97–99). Multi-omics approaches can associate transcriptional states with epigenetic signatures and therefore have great potential for revealing causal regulatory networks. In addition, they allow for linking of *cis*-regulatory elements and target genes, and merging regulatory networks with clustering and trajectory inference, which will in turn provide better understanding of cellular identity and lineage relationships.

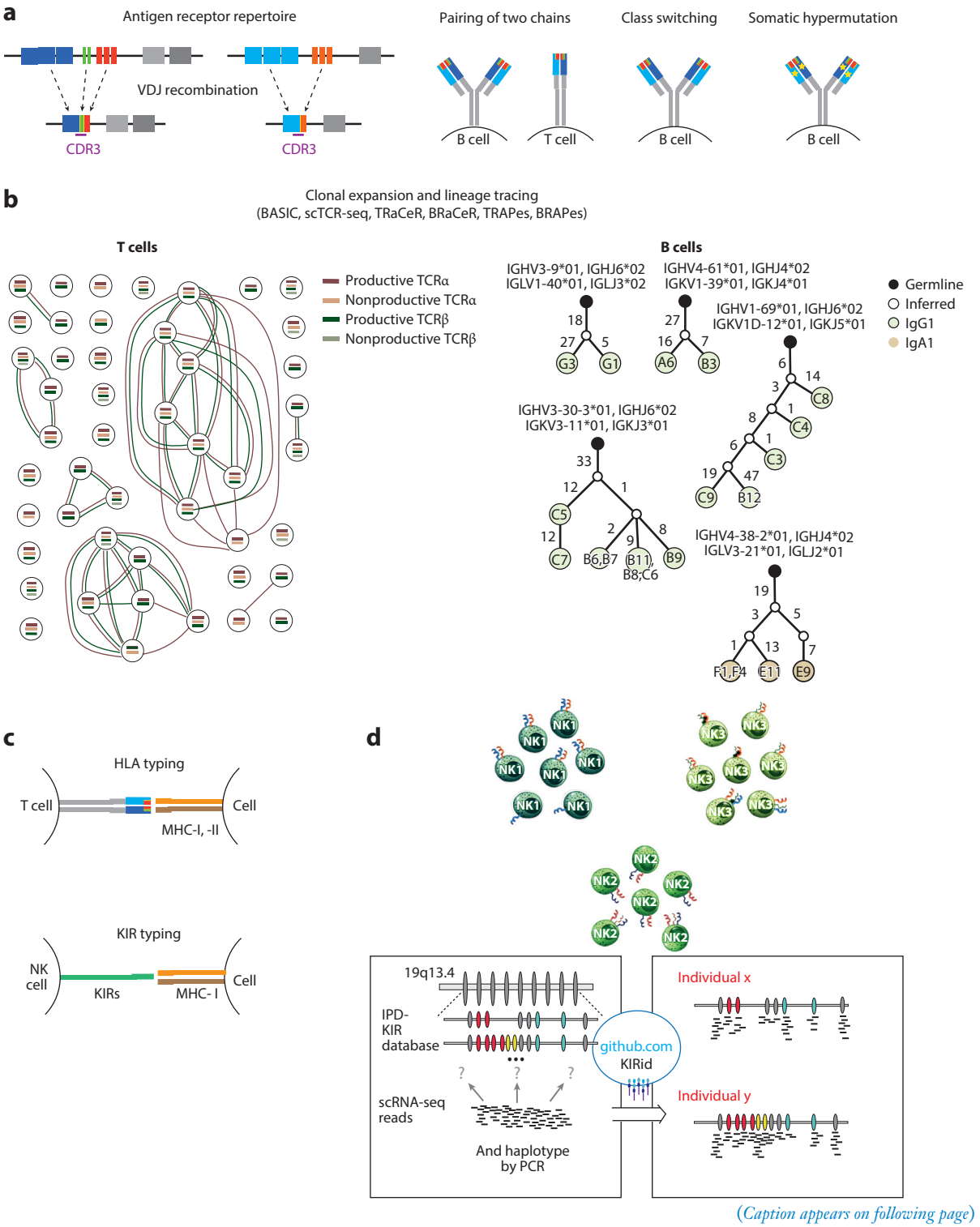
Another approach to infer causal regulatory programs is through perturbation experiments, at the level of either transcription factors or enhancers, to study their effect on the transcriptome. Several recent methods paired single-cell technologies with pooled CRISPR/Cas9 genome editing for introducing a library of genetic perturbations into a population of cells (100–102). In this way, regulatory relationships can be assessed by correlating the transcriptomic or epigenomic phenotype of a cell with a perturbation of a certain regulator. Therefore, these approaches can be a powerful strategy to dissect gene regulatory networks and uncover molecular mechanisms that govern cell fate and function.

ANTIGEN RECEPTOR REPERTOIRE

To cope with the variety of pathogens that we encounter in daily life, the adaptive immune system generates a highly diverse repertoire of antigen receptors. T cells and B cells, the main players of adaptive immunity, acquire antigen specificity by expressing evolutionarily related TCRs and BCRs (also known as immunoglobulins). Antigen receptors have two chains, which are composed of variable (V), diversity (D), and joining (J) segments (**Figure 3*a***). Each of these V, D, and J segments is selected from multiple copies that exist as a genomic array, and this combinatorial selection process confers basal diversity. This junctional diversity, together with pairing of two chains, can lead to an excess of millions of unique antigen receptor pairs. Diversity is further increased by mutational processes at the junction of recombination and pairing between two recombined chains (**Figure 3**). Finally, immunoglobulins undergo multiple rounds of somatic mutation during antigen responses (**Figure 3**). The sequence encoded by V-(D)-J junction is called complementarity determining region 3 (CDR3), which recognizes the antigen peptides presented by the major histocompatibility complex (MHC), thus determining antigen specificity (**Figure 3**). The theoretical diversity of TCR and BCR repertoires can reach beyond 10^{15} chains (103). In reality, bias in V, D, or J gene usage and pairing between two antigen receptor chains limits this diversity, still resulting in around 10^{13} clonotypes. Thus, T cells and B cells are bona fide unique cells in our body, making them great candidates for single-cell approaches.

The immune repertoire contains information about the history and current status of the adaptive immune system. Antigen recognition by T cells and B cells results in the expansion of antigen-specific T cells and B cells, and clonal expansion is often used as a marker of an active immune response. Moreover, analysis of peptide sequences encoded in the clonally expanded CDR3 region can be used to compare the immune response from multiple individuals and to identify a potential antigen.

The importance of immune repertoire profiling has led to the development of numerous methods that have unique advantages and disadvantages. To understand the characteristics of each method, it is worth considering some important aspects in immune repertoire profiling: throughput, accuracy, resolution, and accompanying information on the status of lymphocytes. Throughput is an important aspect of immune profiling due to the great diversity within immune repertoires. The number of lymphocytes in the human body is estimated to be around 10^{11} (104), which exceeds the number of sequence reads that can be obtained by a single run of next-generation sequencing. Thus, the right coverage should be chosen for each question. If the goal is to find the enriched antigen receptor repertoire during an active immune response, a low-throughput approach can be used. On the other hand, high-throughput methods are required for comparing the repertoire diversity between individuals. Another important aspect is accuracy and resolution of the method. For example, cDNA-based techniques are generally considered to more accurately measure abundance of repertoire compared to genomic DNA (gDNA)-based methods, as they start from a larger number of mRNA copies, therefore decreasing PCR bias. UMIs often included in the library preparation step also improve the accuracy of quantification.



(*Caption appears on following page*)

Figure 3 (*Figure appears on preceding page*)

Schematic of immune receptor diversity achieved by VDJ recombination. (*a*) Pairing of two antigen receptor chains, class switching, and somatic hypermutation. The stars represent somatic mutations. (*b*) The diversity of immune antigen receptor repertoires can be studied by computational reconstitution [BASIC (106), scTCR-seq (111), TRaCeR (112), BRaCeR (113), TRAPes (114), BRAPes (115)] using single-cell sequencing data. (*c*) Populational diversity in HLA types and KIR receptors. (*d*) Schematic diagram showing variability in expression profiles of KIR receptors on a single-cell level, across different NK types, and a workflow for the KIRid method (see **https://github.com/Teichlab/KIRid**). Abbreviations: D, diversity; IPD, Immuno Polymorphism Database; J, joining; KIR, killer cell immunoglobulin-like receptor; MHC, major histocompatibility complex; NK, natural killer; PCR, polymerase chain reaction; V, variable.

In terms of resolution, bulk sequencing approaches provide CDR3 sequences, but only single-cell methods obtain information on both chains of antigen receptor pairs, which is necessary to accurately define clonotypes and model antigen specificity. Finally, as lymphocytes are highly plastic and exist in multiple states, it is useful to match the cell status and antigen receptor information. Cell state information can be obtained by FACS-sorting cells prior to repertoire analysis or by combined acquisition of cell state information together with repertoire analysis.

A recent development in single-cell-based immune repertoire analysis methods offers multiple advantages over traditional bulk cDNA- or gDNA-based methods. To obtain paired antigen receptor sequences from single cells, pioneering studies have applied emulsion PCR, which compartmentalizes each cell, or bead-capturing mRNA derived from a single cell in a lipid droplet (105, 106). Reverse transcription and linkage PCR are then performed within droplets, resulting in a fused PCR product containing the sequences of both TCR or BCR chains, allowing the identification of paired sequence information for thousands of cells per sample. In another study, Han et al. (107) combined TCR sequencing with cellular phenotyping by sorting single cells into multiwell plates and adding primers specific to several genes important for T cell identity.

The combination of repertoire information with cellular transcriptome is now readily available through scRNA-seq methods with full-length mRNA coverage (108) showing shared clonality between different cell fates (50), or local clonal expansion in specific organs (37). While full-length coverage methods offer greater depth of transcriptome information, enabling accurate analysis of lymphocyte subtypes, they suffer from relatively lower throughput of, at most, thousands of cells analyzed per study. Droplet-based scRNA-seq techniques offer greater throughput, easily reaching up to 10^4–10^5 cells per study. However, as droplet-based methods are based on barcode-tagging termini of mRNA, only terminal mRNA fragments are obtained, limiting the coverage of the CDR3 region. To overcome this issue, several methods have been developed to increase the coverage of the CDR3 region in droplet-based scRNA-seq. One such approach utilizes in-drop scRNA-seq to add cell-unique barcodes to the 3′ end of mRNA and then enriches TCR sequences by performing RT-PCR with primer sets specific for the V regions of TCRs (109). This has been used to identify paired TCR clones within Treg cell populations. In another widely adopted technique developed by 10× Genomics, the 5′ ends of mRNA are tagged with barcoded template-switch-oligo, and PCR is performed with TCR- or BCR-specific primers. As V-D-J segments are located at the 5′ end of mRNA, this method is highly efficient in capturing the fragment covering this region. This protocol has been applied to study TCR clonality of tumor-infiltrating T cells in a breast cancer model, generating 27,000 paired TCR sequences combined with the T cell transcriptome, showing that expanded clones share similar phenotypic states (110).

As high-throughput single-cell approaches are expanded to incorporate measurement of surface proteins (CITE-seq) and chromatin accessibility (scATAC-seq), immune repertoire profiling has also been combined with these new layers of information. For example, DNA-conjugated antibody labeling has been combined with specific enrichment of TCR sequence, allowing for identification of the $CD4^+$ and $CD8^+$ T cell–associated TCR repertoire from more than a thousand

single cells and revealing bias in TCR repertoire in different T cell types (111–113). Notably, this study confined the readout to a few molecules (CD4, CD8, and TCR), which dramatically reduces the cost of sequencing and allows for increased throughput. Satpathy et al. (14) developed the technique named T-ATAC-seq, which combines a TCR-specific reverse transcription reaction with ATAC-seq on a microfluidics platform. They applied this technique to T cell leukemia and identified malignancy-associated cancer-clone-specific epigenetic patterns.

Increased throughput of new single-cell techniques for immune repertoire analysis led to rapid development of computational analytic tools. As most single-cell transcriptomics platforms generate short reads and antigen receptor sequences that are subject to mutational processes, de novo assembly is required to reconstruct the full sequence. To achieve this, short reads are first mapped onto V, D, and J genes annotated by the IMGT database (**http://www.imgt.org/genedb/**), and the aligned reads are used to reconstruct the full-length transcript. There are a number of tools providing this functionality, such as BASIC, scTCR-seq, TRaCeR, BRaCeR, TRAPes, and BRAPes (108, 114–118) (**Figure 3*b***). Analytical frameworks are also being developed to systematically combine the information of clonality and gene expression. For example, Zhang et al. (58) defined four indices to measure the enrichment, clonal expansion, tissue migration, and state transition of T cell clusters in colorectal cancer.

In addition to the adaptive immune repertoire, killer cell immunoglobulin-like receptors (KIRs) are a family of polymorphic activating and inhibitory receptors expressed on natural killer (NK) cells and a subset of T cells (119). They regulate the development, activation, and tolerance of the NK cells by interacting with MHC class I molecules (HLA-A, -B, and -C) (**Figure 3**). Unlike T cells, these receptors are germline encoded and do not undergo somatic gene rearrangements. Both human KIRs and their ligands, MHC class I molecules, are highly polymorphic, and several studies have demonstrated associations between certain combinations of KIR and HLA genes and susceptibility to diseases, including pregnancy disorders, autoimmune diseases, viral infections, and cancers (120, 121). The extensive KIR diversity poses a challenge for accurate quantification of expression, and to address this, we developed KIRid, a method that uses full-length transcript Smart-seq2 data to map the single-cell reads of each donor to the corresponding donor-specific reference of KIR alleles (37).

RECONSTRUCTING SPATIAL ENVIRONMENTS

The exponential growth of single-cell transcriptomics methods (122) has provided a unique opportunity to analyze the expression of multiple cell types in diverse tissues and systematically decode intercellular communication networks. By measuring the expression of known ligands and receptors (123, 124) in the distinct cell types identified by single-cell transcriptomics, we and others have started to generate potential cell-cell interaction networks (37, 39, 125–127). The high accessibility and unbiased nature of single-cell technologies allow the identification of potentially interacting cells that can be validated by orthogonal spatial and functional methods afterward.

Other approaches, such as ProximID (128) and Paired-seq (129), profile cell proximity by making use of readouts representing more than one cell. These methods rely on the optimization of enzymatic tissue digestion protocols and the selection of paired cells prior to sequencing. They have proven effective in the study of interactions of endothelial cells in the liver and hematopoietic progenitors in the bone marrow. However, resolving the accurate tissue architecture and interactions between all neighbors requires the use of spatial-resolved methods.

One approach to integrate spatial data with single-cell transcriptomics is to create a spatial reference using landmark genes (130–132). Genes that are differentially expressed in the tissue space can be obtained from legacy knowledge or by combining orthogonal techniques like laser

capture microdissection (LCM) of tissue (133). Although very useful, differentially expressed genes within the tissue are not always known, and LCM methods can be laborious and dependent on specific infrastructure. A recent method introduced a different approach for de novo spatial reconstruction, without reliance on an existing reference atlas (134). In recent years, the sensitivity and high throughput of spatial imaging and sequencing methods have increased notably (reviewed in 135–137). In this section, we summarize the recent advances in the field and the strengths and weaknesses of the new methods.

Imaging the Transcriptome

The classical method of measuring gene expression in their native context is smFISH (138, 139). This method relies on specific probes that recognize cellular RNA directly in the tissue at a subcellular resolution. The high sensitivity of this method easily allows for automated measuring of lowly expressed genes, such as those encoding transcription factors, when combined with computational tools for cellular segmentation and quantification. However, it is often the case that only a few markers can be assessed in parallel, limiting its use for determining cell-cell communication.

In order to increase the number of genes detected, DNA probes with a specific combination of fluorophores (combinatorial labeling) can be used in conjunction with superresolution imaging (140). Sequential hybridization offers a good alternative for multiplexing. Here, the RNA barcode is generated over time by consecutive stainings and rounds of hybridization and imaging (141). Error-correction encoding schemes are crucial in improving the detection of the signal and the alignment of images in distinct hybridization rounds, enabling imaging of 100 to 1,000 expressed genes in individual cells in a high-throughput manner (142, 143). The plexing and accuracy of these technologies have now been escalated to genome level, and it is now possible to detect more than 10,000 genes in parallel using a standard confocal microscope (144). These improvements offer unbiased analysis of cell expression directly in tissue but require sophisticated data analysis and statistical methods to interpret the data.

Sequencing the Transcriptome in Space

Sequencing methods capture all forms of single nucleotide variation, which may be useful to observe the effect of somatic mutations on gene expression. In in situ sequencing methods, enzymatic reactions to retrotranscribe RNA and amplify and sequence cDNA are performed directly in the tissue using sequencing-by-ligation chemistry (145, 146). Here, combinations of nucleotides are associated with a fluorophore that is detected when hybridized in the amplified sequence. Some of these methods, such as fluorescent in situ RNA sequencing (FISSEQ) (146), demonstrate genome-wide scalability; however, their implementation for analysis of complex tissues is challenging. Spatially resolved transcript amplicon readout mapping (STAR-map) makes use of hydrogel-tissue chemistry and in situ sequencing to map more than 1,000 genes in 3D-intact tissue (147). This promising technology enables quantification of the distribution and cell contacts in their native tissue context.

An alternative to these methods is spatial transcriptomics, where RNA is diffused from the tissue to spatial barcodes positioned on imprinted slides (148). These spatial barcodes act as a template for cDNA synthesis, and sequencing is performed outside the tissue. Recently, slides have been replaced by barcoded beads, which enhances the resolution to a level close to single-cell (149, 150). Barcoded beads are decoded by sequential hybridization once deposited on the array, making the implementation of this method dependent on a good imaging setup.

A promising novel method to measure the proximity of individual molecules without using optics has recently emerged (151). Here, transcripts are tagged in situ with unique randomized

barcodes where cDNA is amplified with overlap-extension primers and proximal molecules are concatenated. This technology requires a good computational setup, and its suitability for complex tissues is yet to be demonstrated.

Imaging Proteomes

Proteins are the final, functional product of gene expression, and measuring their presence in situ is extremely useful to detect processes dependent on posttranscriptional modifications like cell signaling. However, multiplexed protein methods are challenging as they rely on the detection of the protein by highly specific antibodies that are not always available. The two approaches that are currently being used for multiplexing proteins are mass cytometry imaging and multiplexed fluorescence imaging (for a detailed review see 137, 152).

Mass cytometry imaging is based on heavy metal isotope–conjugated antibodies that possess a unique atomic mass that can be measured and quantified using specific equipment. In contrast to the original methods where measurement happens in cell suspensions (153, 154), mass cytometry imaging quantifies protein expression in its native tissue context. These methods rely on expensive equipment that requires specialized training. Affordable alternatives are methods using DNA-barcoded antibodies (155, 156). Here, immunostaining is only performed once, and identities are revealed in situ by incorporating nucleotides conjugated with fluorophores.

Multiplexed fluorescence imaging is based on the detection of fluorescent proteins or fluorophore-conjugated antibodies against surface or intracellular proteins. Although limited to simultaneous detection of four to five labels, they can be used to visualize a cell type of interest within tissues. An elegant example is the application of fluorescence microscopy by Stewart et al. (157). In this study, using scRNA-seq on human kidney combined with cell-cell interaction analysis, we observed epithelium-immune cross talk that orchestrated recruitment of antibacterial macrophages and neutrophils to regions most vulnerable to infections. Moreover, using multiplexed fluorescence imaging, we validated the zonation of $CD11b^+$ $LysM^+$ neutrophils to the pelvis of the nephron during urinary pathogenic *Escherichia coli* infection in mice, where they are ideally located to tackle the infection (**Figure 4**) (157). Alternative single-cell methods to visualize or sequence the transcriptome at spatial resolution as described above can similarly be applied or used in combination to assess immune cells in situ (summarized in **Table 2**).

Spatial methods integrated with single-cell transcriptomics will help to build 3D maps on an unprecedented scale. Cell identity and function depend on their surroundings; herein, spatial methods will enable a better definition of cells and tissue organization.

IMMUNE CELLS IN HEALTHY TISSUE

Tissue-resident immune cells not only provide surveillance and self-defense mechanisms but also contribute to tissue homeostasis, development, and repair (158, 159). Macrophages are an obvious example of the capability of immune cells to adapt to tissues, presenting a large spectrum of phenotypes that are dependent on the tissue context. For example, osteoclasts are macrophages specialized in bone resorption, and microglial cells are brain macrophages that support neural signaling (160). scRNA-seq of whole organisms has been instructive in defining the unique signatures of tissue-resident immune cells in adult (161, 162) and geriatric life (163, 164). However, mapping of all cells in the human body requires a well-coordinated, global effort. The Human Cell Atlas initiative (165), an international consortium that aims to map all cells in the human body, has already generated maps for whole tissues (37, 39, 166). The development of novel computational and statistical methods to facilitate the integration of data sets is crucial for outlining the expression profiles that will ultimately define the adaptation of cells to tissues.

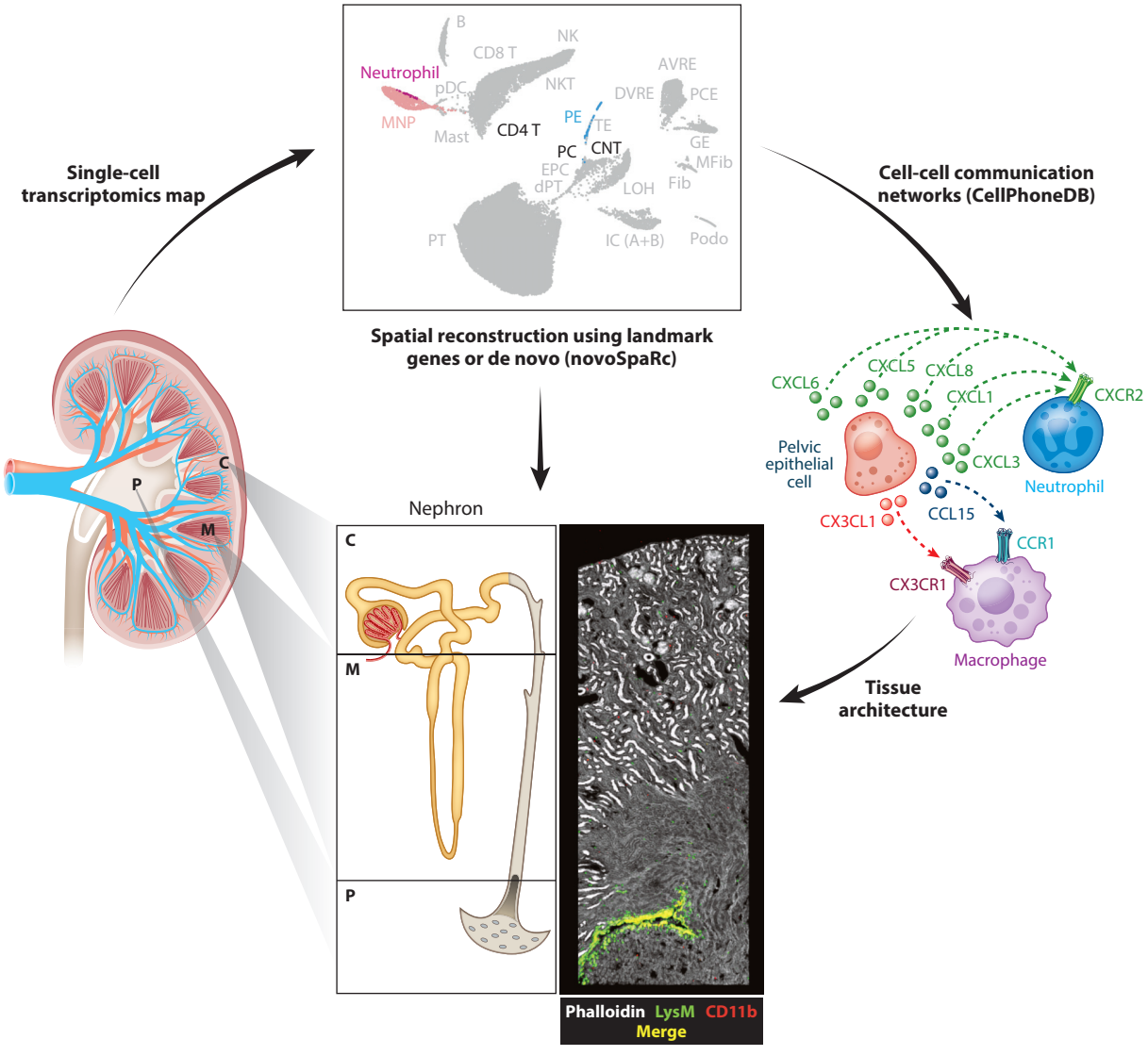


Figure 4

Single-cell spatial approaches to study immune cell zonation. Shown are (*top*) a UMAP plot illustrating the broad cell populations in the human mature kidney; (*right*) selected ligand-receptor interactions between neutrophils, antibacterial macrophages (part of the MNP cluster), and pelvic epithelium cells, inferred using CellPhoneDB (37, 124); (*bottom* and *left*) a diagram of the kidney nephron, divided into cortex, medulla, and pelvis; and confocal microscopy images of kidney from cortex to pelvis of neutrophil markers LysM and CD11b in a LysMGFP transgenic mouse with urinary pathogenic *Escherichia coli* (UPEC) infection. Fluorescence shows phalloidin (*white*), LysM (*green*), and anti-CD11b (*red*), and merged LysM and anti-CD11b (*yellow*). Abbreviations: AVRE, ascending vasa recta endothelium; C, cortex; CNT, connecting tubule; dPT, distinct proximal tubule; DVRE, descending vasa recta endothelium; EPC, epithelial progenitor cell; Fib, fibroblast; GE, glomerular endothelium; IC (A+B), intercalated cell (types A and B); LOH, loop of Henle; M, medulla; MFib, myofibroblast; MNP, mononuclear phagocyte; NK, natural killer cell; NKT, natural killer T cell; P, pelvis; PC, principal cell; PCE, peritubular capillary endothelium; pDC, plasmacytoid dendritic cell; PE, pelvic epithelium; Podo, podocyte; PT, proximal tubule; TE, transitional epithelium of ureter. Adapted from 157.

Table 2 Summary of single-cell spatial approaches

Single-cell spatial approach	Technology	Ref.
Imaging the transcriptome	smFISH, DNA microscopy, MERFISH, seq-FISH	138–144
Sequencing the transcriptome	Spatial transcriptomics, FISSEQ, STAR-map, Slide-seq, HDST	145–151
Imaging the proteome	Mass cytometry imaging, multiplexed fluorescence microscopy	Reviewed in 137, 152; 153–156

Abbreviations: FISH, fluorescent in situ hybridization; FISSEQ, fluorescent in situ RNA sequencing; HDST, high-density spatial transcriptomics; MERFISH, multiplexed error-robust fluorescence in situ hybridization; seq-FISH, sequential FISH; smFISH, single-molecule FISH; STAR-map, spatially resolved transcript amplicon readout mapping.

In this section we summarize studies where scRNA-seq analysis combined with orthogonal antibody-based methods and cell-fate tracing has made it possible to define novel populations, origins, and functions of immune cells in the steady state across the human life span.

A Deep Dive into Mononuclear Phagocytes

Cell identity and function are a reflection of distinct expression programs, and therefore, unbiased approaches such as scRNA-seq are used to define new cell types. scRNA-seq and orthogonal methods such as flow cytometry were used to generate a better classification of human mononuclear phagocytes (MPs) in peripheral cells and describe the potential ontogeny of MPs in blood (32, 33). These studies revealed novel MP populations and relationships between them, reflecting a need to review the current classification of immune cell types.

To define novel subsets of tissue-resident macrophages and their communication with other nonimmune cells, we and others have performed scRNA-seq in tissues including heart (167), adipose tissue (168), brain (35, 169, 170), and lung (39, 171). The location of tissue-resident immune cells is highly related to their phenotype and function. Therefore, combining imaging methods and single-cell transcriptomics in these tissues is crucial to characterizing novel macrophage subsets. Following this strategy, Chakarov et al. (171) defined two populations of interstitial macrophages in multiple tissues in mice, including lung, heart, fat, and dermis. While one of the populations is in contact with nerves and is specialized in wound healing, repair, and fibrosis, the other sits close to vessels regulating inflammation and tissue infiltration. Following this approach, scRNA-seq in human and mouse has led to the discovery of novel conserved macrophage populations in adipose tissue (168) and brain (170).

Mucosal Immunity of Innate Lymphocytes

Innate lymphocytes comprise a heterogeneous group of lymphocytes that do not express highly variable antigen receptors (i.e., BCRs or TCRs) and are specialized in cytokine production. The five major groups of innate lymphocytes (172)—NK cells, group 1 innate lymphocytes (ILC1s), ILC2s, ILC3s, and lymphoid tissue-inducer cells—mirror the nonspecific functions of the adaptive T cell responses and have roles in mucosal immunity. scRNA-seq of ILCs has provided better understanding of their heterogeneity, proposing a spectrum of peripheral and resident subsets (38, 173, 174). Through pseudotime inference from scRNA-seq data, molecules potentially regulating the conversion of ILC and NK subsets have also been studied (175, 176). Due to the role of the ILC in mucosal immunity and NK subsets in cancer therapy, these findings have relevant clinical implications.

Decidual NK (dNK) cells are a specialized population of resident NK cells in the decidua, the mucosal lining of the uterus during pregnancy, with unique morphology and expression of

surface receptors (177, 178). Our atlas of the maternal-fetal interface during early pregnancy in humans has defined three novel populations of dNK cells (dNK1, dNK2, and dNK3), and their markers and morphology (37). By using CellPhoneDB (124), our novel computational framework for studying ligand-receptor interactions from transcriptomics data, we have defined the unique interactions between dNK cells and other fetal and maternal cells, as well as their potential role in immunomodulation and trophoblast invasion.

Fine-Tuning Immune Cell Tissue Adaptation

Single-cell transcriptomics has also been used to delineate the heterogeneity of Treg cells and their adaptation to tissue environments (54, 55, 109). Using pseudotime ordering, Miragaia et al. (54) defined the trajectories of T memory and Treg cells from lymphoid to peripheral tissues and the genes modulating this tissue adaptation. With the generation of our recent human gut atlas (unpublished data set; 178a), we have now been able to study conserved mechanisms mediating the adaptation and acquisition of suppressive phenotype of Treg cells in the human gut.

A recent publication focusing on blood from supercentenarians—humans older than 110 years—has revealed changes in the composition of the adaptive immune response and T cell repertoires associated with aging (179). Specifically, the authors show a shift in the B/T cell ratio, with an increased clonal expansion of cytotoxic $CD4^+$ T cells. Interestingly, aging has also recently been associated with an infiltration of T cells in the brain, which potentially relates to the reduction in the stem cell potential of neural stem cells (180).

Reconstructing the Origin of Immune Cells

Mapping the development of the immune cells is highly relevant for basic biology and has clinical implications. Some blood cancers and immunodeficiencies originate in aberrant immune progenitors. In addition, understanding the molecules driving the differentiation of immune cells may help to design better engineered immune cells for immunotherapy. Bone marrow is the main hematopoietic organ after the second trimester of fetal development and in adult life. Therefore, delineating the heterogeneity and differentiation potential of hematopoietic progenitors (181, 182) and their interactions within tissue environments (183) has been a priority in the field of single-cell transcriptomics. The trajectory of progenitors from human bone marrow has also been profiled in humans using scRNA-seq and complemented with functional assays to measure the potential differentiation of progenitors in vitro and validate computational predictions (184).

Combination of single-cell transcriptomics, mass cytometry, and in vivo fate mapping methods has been useful in defining the ontogeny of macrophages and dendritic cells. For example, using mouse models, the dual myeloid and lymphoid origin of plasmacytoid dendritic cells (pDCs) was proven, with the lymphoid origin being most prevalent for adult pDCs (185, 186). Our recent single-cell atlas of hematopoietic progenitors in the liver during the first and early second trimesters of development suggests human pDCs also appear to have dual lymphoid and myeloid origins in humans (187). We (187) combined a single-cell transcriptomic atlas of early development of fetal liver with functional assays to validate the potential of hematopoietic stem cells to differentiate into lymphoid and myeloid cells, revealing a distinct intrinsic modulation across the gestational stage of life. Our hematopoietic atlas includes matched peripheral organs to study the molecular mechanisms of tissue seeding and adaptation. Light sheet and confocal microscopy imaging performed on fetal tissues using markers from scRNA-seq analysis revealed erythroblasts in fetal skin, suggestive of physiological erythropoiesis in the developing skin.

The results mentioned above point toward a dynamic composition of the immune component over time—through development, adult life, and aging—and space—peripheral immune cells and

tissue-resident cells. Integrated computational analysis of the data sets will give us a holistic view of the mechanisms involved in tissue adaptation and their conservation between species.

STUDYING IMMUNE CELLS DURING DISEASE

Immune cell responses during inflammation, autoimmunity, and infection are characterized by heterogeneity and cellular plasticity—features that are particularly well resolved through single-cell methods. In this section we look more closely at key examples of how single-cell approaches have been and can be applied to these diseases, particularly to further our understanding of the contribution of immune cells to pathology and protection, and host cell–pathogen relationships.

Relationship Between Host Cells and Pathogens

An area in which scRNA-seq has proven highly informative is in understanding the relationship between host cells and viruses or intracellular bacteria. Several studies have looked for the presence of virus within host cells using fluorescence techniques to monitor virus behavior in the infected cells (188, 189). In particular, the kinetics of poliovirus in individual cells was explored, revealing variation in virus replication kinetics between individual cells and that host cell population heterogeneity influences the outcome of a viral infection (190). More recent work by Steuerman et al. (191) utilized scRNA-seq to investigate heterogeneity in the response of lung tissue cells to influenza infection in mice. This approach was able to simultaneously measure host and viral transcriptome in the same cell, allowing the authors to determine bystander cell heterogeneity versus infected-cell heterogeneity. scRNA-seq could similarly be applied to viruses with polyadenylated transcriptomes, including both RNA viruses (influenza, Ebola, measles) and DNA viruses (herpesviruses, adenoviruses, poxviruses) (192).

To focus on how immune cells specifically respond during infection, Martin-Gayo et al. (193) applied scRNA-seq to conventional dendritic cells (cDCs) isolated from elite controllers of HIV and other infected individuals to understand if these cells contributed to an improved response. Mohammadi et al. (194) investigated the cell heterogeneity of HIV-infected cells during latent and activated stages in a primary model of latently infected $CD4^+$ T cells in an effort to understand susceptibility of $CD4^+$ T cells to HIV infection. In our own work looking at T helper responses to malaria, we applied Smartseq2 to $CD4^+$ T cells during in vivo blood-stage *Plasmodium* infection in mice (50). An important advantage of applying a single-cell approach here is that, using computational analysis, we were able to reconstruct the cellular response trajectory and demonstrate a bifurcation into two effector cell types (Th1 or Tfh). By observing shared expression of TCR sequences (also captured by scRNA-seq) and expression of signaling molecules across this trajectory, we inferred that effector fate of $CD4^+$ T cells is not predefined in the naive state, but the result of continued cell-cell signals during the infection (50).

On a more general level, scRNA-seq has been applied to the evolutionary arms race between viruses and host cells in mammals by Hagai et al. (195). This extensive investigation of fibroblasts from different species revealed that rapidly diverging genes between species show higher levels of variability in their expression across cells than genes that diverge more slowly. Moreover, they showed that expression of regulators of the immune response, i.e., transcription factors, are relatively conserved among species, likely owing to their roles in multiple contexts and pathways. Cytokines, on the other hand, showed divergent expression between species because they have fewer constraints imposed by intracellular interactions or additional nonimmune functions.

Inflammation

To generate a better appreciation of the complex network of cellular responses underlying inflammatory diseases, a number of studies have performed scRNA-seq on unfractionated cells, from diseased tissue to healthy control tissue. As an example, single-cell RNA-seq of biopsies from asthmatic lung versus healthy lung tissues revealed that a pathogenic Th2 cell state was identified only in asthma samples, as well as a novel mucous ciliated cell state that gives rise to mucous cell hyperplasia (39). Additionally, cell-cell communication network analysis of the two cohorts showed a shift from airway structural cell communication in healthy lungs to a Th2-dominated interactome in asthmatic lungs.

In a similar study setup, Smillie et al. (196) compared epithelial, stromal, and immune cells from colonic mucosa of terminal ileum of healthy subjects and individuals with ulcerative colitis (UC). Using droplet-based single-cell RNA-seq, they performed cell type–specific UC genome-wide association studies (GWASs) and identified risk alleles that are coregulated in relatively few gene modules, and nominated and inferred putative functions for UC risk genes across all GWAS loci.

scRNA-seq has also been applied to renal biopsies and matching skin biopsies from patients with lupus nephritis and healthy controls. Identification of type I interferon response signatures in tubular cells and keratinocytes distinguished patients with lupus nephritis from healthy control subjects, suggesting these signatures could be used as a potential source of diagnostic and prognostic markers of renal disease (197).

Autoimmunity

In the area of autoimmune research, Zhang et al. (198) combined scRNA-seq with bulk RNA-seq, mass cytometry, and flow cytometry to profile immune cells from synovial tissue from patients with rheumatoid arthritis or osteoarthritis. By integrating these methods, they identified four transcriptionally distinct fibroblast populations and supported their identity at the protein level. scRNA-seq has also been used to determine a systemic autoimmune transcriptional profile of circulating $CD1c^+$ conventional DCs in patients with rheumatoid arthritis compared with the equivalent population in healthy individuals, and to show that the frequency of these cells is directly correlated with the extent of disease activity (199). Another recent study combined fate-mapping methods, scRNA-seq, and three-dimensional light-sheet fluorescence microscopy. This allowed the authors to characterize the composition, origin, and dynamics of diverse subsets of tissue-resident and monocyte-derived synovial macrophages in healthy and inflamed joints in arthritis (200). They revealed that a population of $CX3CR^+$ macrophages, derived from a tissue-resident $CX3CR1^-$ population, forms an immunological barrier at the synovial lining that protects intra-articular structures and controls the onset of inflammation. The development of high-throughput BCR and TCR sequencing at the single-cell level has opened up the possibility to study lymphocytes that respond to self-antigen in autoimmunity. In a particularly interesting study, the dual expression of a TCR and a BCR (and other defining markers of T and B cell lineages) was identified in peripheral blood lymphocytes of human donors (201). In patients with type 1 diabetes, these dual-expressing lymphocytes were enriched for one clonotype with optimal binding register for diabetogenic HLA-DQ8, hinting at its pathogenic contribution. Recent advances in TCR/BCR sequencing with paired transcriptional information at single-cell resolution will likely be a prominent feature of future autoimmunity studies.

Cancer

Cancer is a complex disease characterized by intratumoral heterogeneity reflected at genomic, transcriptomic, and proteomic levels. Stochastic accumulation of somatic mutations results in

increasing genetic diversity and subclonal populations with distinct genotypes and phenotypic differences. The somatic mutations driving cancer progression serve as tumor-associated antigens targetable by immune cells. Moreover, tumor cells interact with nonmalignant cells in the surrounding microenvironment where selective pressures operating in different tumor regions help create a complex ecosystem characterized by dynamic interactions between diverse cell types, including malignant, immune, and stromal cells. These interaction networks contribute to shaping cancer progression and are crucial for patient outcome.

Traditionally, cancer research was approached at the whole-population level. However, bulk technologies only measure the average profile, and the transcriptomics profiles of these cells are intermingled, making it difficult to deconvolve the individual signals. Emerging single-cell technologies have begun to dissect the tumor heterogeneity at various levels, from genotype to phenotype.

Using single-cell transcriptomics, the immune landscapes of diverse cancer types, including glioblastoma (202, 203), melanoma (203), head and neck (126, 203), colorectal (204), kidney (205, 206), breast (110, 207), liver (57), and lung (57, 208, 209), have been dissected and analyzed. These studies enabled characterization of tumor-infiltrating immune cell types and states, as well as pathways involved in tumor immunosuppression and invasion. T cells, which can recognize tumor antigens and destroy cancer cells in a targeted way, have been in the spotlight of single-cell studies of several cancer types. Pioneering work in glioblastoma demonstrated the potential to distinguish tumor cells from nonmalignant infiltrating cells in the tumor microenvironment using inferred somatic alterations from scRNA-seq data (202). Building on this work, Tirosh et al. (203) analyzed malignant and nonmalignant cells from 19 melanoma patients and highlighted cell-cell communication between cancer and stromal cells with implications for both immune and targeted therapies. In addition, their analysis revealed T cell exhaustion programs and their clonal expansion. Similar findings were observed in hepatocellular carcinoma, where Zheng et al. (57) identified 11 distinct T cell subsets, including enrichment of infiltrating Treg cells and clonal expansion of exhausted $CD8^+$ T cells. Azizi et al. (110) showed that the greatest immune cell diversity was tissue specific and that differences in phenotypes—specifically attributed to activation, terminal differentiation, and hypoxic response—contributed most to this diversity between healthy and cancerous tissues.

Although less utilized compared to scRNA-seq, single-cell genome sequencing has been used to track clonal dynamics and infer evolutionary histories of diverse cancer types. One of the challenges in single-cell genome analyses is the amplification of a small amount of genetic material to reach the detection threshold level. However, recent advances in whole-genome amplification have achieved high coverage, low false-positive rates, and uniform amplification and enabled detection of both single-nucleotide polymorphisms and copy-number variations at a single-cell resolution (210).

A number of studies investigated the diversity and evolution of single cancer cells in multiple cancer types [breast (211, 212), kidney (213), bladder (214), myeloproliferative neoplasm (215), acute lymphoblastic leukemia (216), colon (217)] and revealed different evolutionary models and dynamics of cancer progression. Furthermore, a recent study (218) combined genetic, epigenetic, transcriptomic, and functional analysis of colorectal cancer organoids and showed that the genetic heterogeneity during cancer evolution is followed by diversity in methylation and transcriptome states and responses to therapeutics. By reconstructing the evolutionary dynamics and clonal structure, single-cell studies hold the potential to address important questions, such as which subclones are immunogenic, which have the potential to become invasive, and which will confer resistance to specific drugs.

Recent progress in single-cell technologies has enabled simultaneous measurement of the transcriptome and genome (219–221) and/or protein (222) from the same cells. In addition,

certain spatial technologies allow for potential scaling up to whole-genome coverage with single-nucleotide resolution and parallel detection of expression profiles and in situ mutation detection, while retaining spatial information (146). Integration of genotype with phenotypic measurements, as well as intracellular and intercellular spatial information will provide important new insights into cancer development and treatment response.

TRANSLATION OF SINGLE-CELL FINDINGS TO THE CLINIC

Complex diseases involve altered behaviors across multiple cell types residing in genetically and phenotypically diverse states. Single-cell technologies, by enabling detailed characterization of cell types and states and pathways associated with human diseases, can have a profound effect on translational applications. To date, clinical applications have mostly been focused on cancer immunotherapy, for investigating molecular mechanisms of drug efficacy and resistance, as well as discovery and development of novel therapeutic targets. However, the underlying principles could be applicable to other complex diseases.

Checkpoint blockade immunotherapy, using antibodies against immune checkpoint molecules to reactivate the suppressed immune system, have transformed the treatment landscape of a number of cancer types, especially melanoma (223). However, the efficacy of treatment varies between patients, raising the necessity to identify factors governing resistance. By dissecting the diverse cancer microenvironment, single-cell methods, combined with carefully designed study cohorts, have shed light onto the immunosuppressive mechanisms operating in diverse cancer types.

Jerby-Arnon et al. (224) utilized single-cell RNA-seq data to deconvolute the large-scale bulk RNA-seq data and identify the cancer gene expression signature that correlates with T cell abundance in melanoma samples. This signature can be used as a predictive marker for the resistance to immune checkpoint therapy. The authors identified CDK4/6 inhibitor as a potent repressor of this resistance program and showed the efficacy of combinatorial treatment of CDK4/6 with immune checkpoint therapy in a mouse model. Sade-Feldman et al. (225) also applied scRNA-seq to melanoma patient samples, focusing on immune cell populations. By comparing gene expression profiles of $CD8^+$ T cells between responders and nonresponders to immune checkpoint therapy, they identified TCR7 as a predictive marker for good clinical outcome and CD39 as a marker for exhaustion. Moreover, inhibition of CD39 activity in combination with anti-TIM3 antibody resulted in reduced tumor growth and increased survival of mice with B16-F10 tumors, demonstrating the power of single-cell transcriptomics to provide effective therapeutic combinations and biomarkers for predicting patient responses to immunotherapies.

PERSPECTIVE

The complexity of the immune system for effective responses to pathogens and preventing and eliminating disease is regulated by an intricate network of diverse and highly plastic cells and cell-cell interactions. Single-cell technologies have provided tools for generating detailed cellular maps of immune cells, in both homeostasis and pathological conditions at an unprecedented resolution. Combined with analytical methods and lineage-tracing techniques, these approaches have helped delineate lineage hierarchies and identify states at which cell fate decisions are made and regulatory elements that drive these decisions. Here, we have highlighted recent technological and methodological advances, as well as studies that have applied these approaches to address fundamental questions in immunology.

Moving forward, emerging multi-omics approaches will provide comprehensive profiles of transcriptome, epigenome, and proteome, allowing for a more holistic analysis of cells, including

details of their lineage, current behaviors, and developmental/differentiation potential. Finally, advances in spatial methods will enable quantitative and phenotypic description of cells in their tissue context and study of their communication with neighboring cells. Together, these integrated strategies will give us a more complete view of the essential immune processes and deepen our understanding of immunological responses in both health and disease.

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