Cell

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Perspective

Single-Cell Genomics: A Stepping Stone for Future Immunology Discoveries

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https://doi.org/10.1016/j.cell.2017.11.011

The immunology field has invested great efforts and ingenuity to characterize the various immune cell types and elucidate their functions. However, accumulating evidence indicates that current technologies and classification schemes are limited in their ability to account for the functional heterogeneity of immune processes. Single-cell genomics hold the potential to revolutionize the way we characterize complex immune cell assemblies and study their spatial organization, dynamics, clonal distribution, pathways, function, and crosstalks. In this Perspective, we consider recent and forthcoming technological and analytical advances in single-cell genomics and the potential impact of those advances on the future of immunology research and immunotherapy.

The immune system is a complex network composed of various interacting cell types and functional states (Figure 1). It is one of the most dynamic and plastic systems in the human body, present in nearly every tissue of the organism, and involved in a wide range of homeostatic activities—from tissue development and remodeling (Wynn et al., 2013) to metabolism and neuronal maintenance (Brestoff and Artis, 2015; Schwartz et al., 2013) to clearance of debris and cells that need to be eliminated (Devitt and Marshall, 2011). Its function or dysfunction is even more pronounced in pathology, where various immune cells play a central role in pathogen and tumor clearance or escape, as well as in metabolic, autoimmune, and neurodegenerative diseases. Immune processes are mediated by the crosstalk between many types of cells—tissue resident as well as circulating immune cells—all interacting in specific micro-environmental contexts while communicating with the local tissue. Characterizing these cellular networks, the participating cell types, their unique pathways, and genes, as well as their interactions and responses to environmental cues, is key to successfully manipulating the immune system in order to harness its unique therapeutic potential (Sharma and Allison, 2015).

Since the 19th century, with efforts pioneered by Ilya Mechnikov, a major focus of immunology has been the characterization and categorization of immune cells into distinct types (Tauber, 2003). Early classification efforts assigned cell types based on morphology and cellular functions. For example, macrophages were associated with phagocytosis, whereas dendritic cells were associated with antigen presentation (van Furth et al., 1972; Steinman and Cohn, 1973). Further technological advances, such as improved microscopy, monoclonal antibodies, new fluorophores, FACS, next generation sequencing technologies, and mass spectrometry, dramatically accelerated immunology research by allowing the association of immune cell types with specific molecular markers, spatial organization, and relationship to cohabiting cells within tissues (Fulwyler, 1965; Köhler and Milstein, 1975; Stoll et al., 2002). A central assumption behind these efforts was that once a cell differentiates and acquires an identity, this identity would dictate its functional state, which could be definitively defined by a small number of molecular determinants.

For such a classification scheme to succeed, a cell's identity must be rigid and unambiguously linked with its marker expression pattern. However, recent findings reveal that commonly used markers do not fully describe the functional diversity of immune types and that cellular identity can be highly plastic and dependent on tissue and environmental contexts (Gordon and Taylor, 2005; Gosselin et al., 2014; Lavin et al., 2014). Therefore, it is nearly impossible to describe the spectrum of possible functional states and gene expression programs of the immune system using only a small number of molecular markers (Hume, 2008; Schroeder, 2010). The question of whether new and better markers could resolve this difficulty, or whether it requires the incorporation of additional experimental and analytical tools, was, until recently, subject to debate.

Application of Single-Cell RNA-Seq in Immunology

Our working hypothesis was that, because of the high connectivity, heterogeneity, and plasticity of the immune system, current technologies were often limited in their abilities to fully characterize the various cell types and states involved in immune cell functions. This stemmed from the setbacks of the then-available molecular profiling tools; these allowed either genome-wide profiling of a population of cells, selected by expression of predefined markers, or detection of a small number of molecular features at the single-cell level. Single-cell RNA-seq seemed to be an ideal solution for immunology research, as it overcomes these previous technological limitations. Our study on unbiased characterization of spleen immune cell populations was the first step towards materializing this vision, demonstrating that single cell RNA-seq is an effective tool for comprehensive cellular

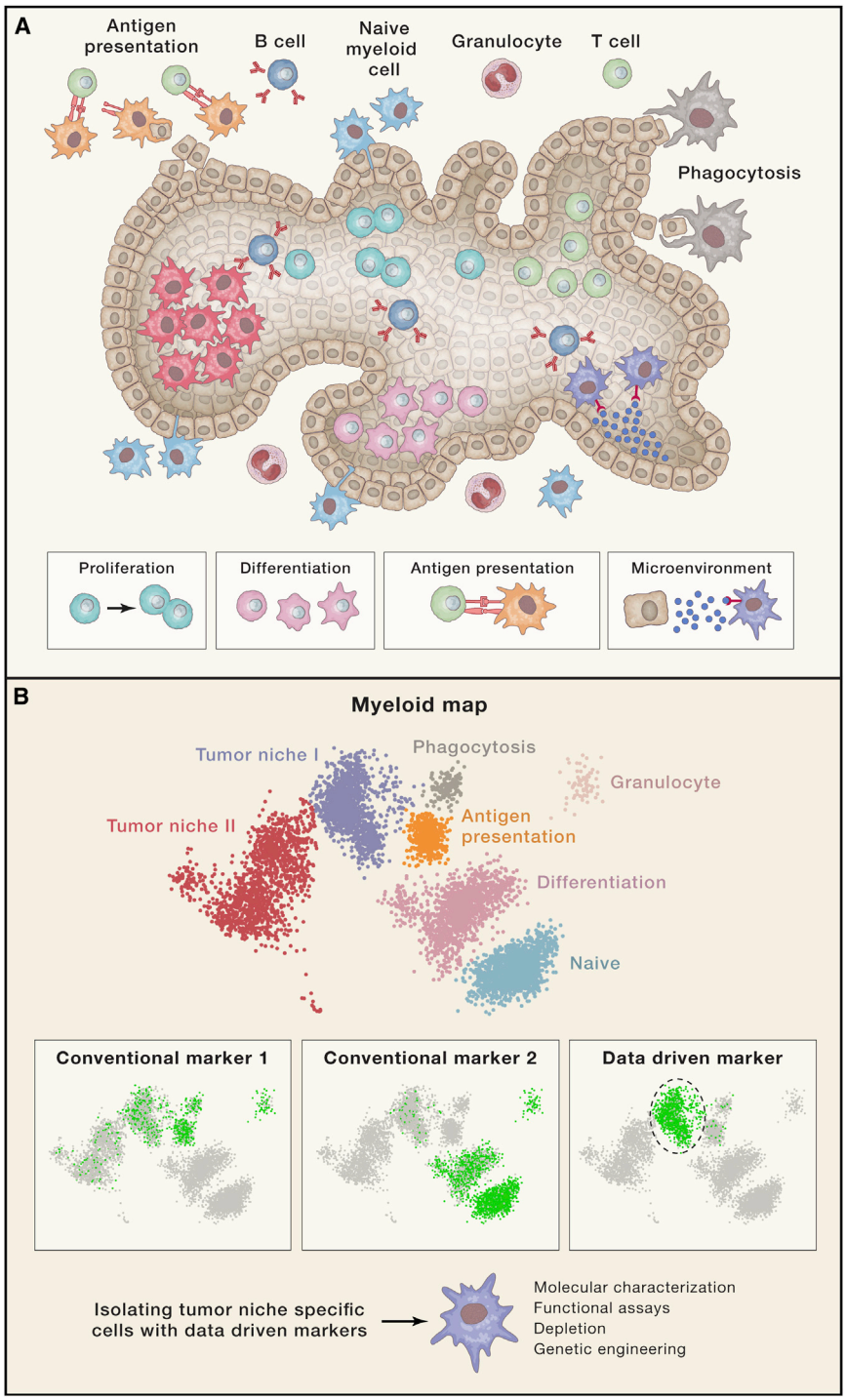

Figure 1. Characterizing Immune Heterogeneity in the Tumor Ecosystem with Single-Cell Genomics

(A) Immune colonization of the tumor niche involves different cell types and functional specifications. Distinct subtypes of lymphoid and myeloid cells are localized to different tumor niches, receive signals from the microenvironment, and participate in proliferation, differentiation, antigen presentation, and phagocytosis.

(B) Holistic single-cell sequencing and analysis of tumor-derived myeloid cells can dissect this complex population into distinct cell types and functional biological processes based on their transcriptome, whereas gating based on conventional surface markers may fail to be cell-type specific. Newly discovered data-driven markers can be used to isolate tumor-specific immune cells for further discovery and intervention studies.

decomposition of complex tissues without the need for predefined markers (Jaitin et al., 2014).

In the last few years, the single-cell genomics field dramatically advanced in overcoming the limiting factors that had previously hindered its utility in immunology research. Reproducibility, throughput, and sensitivity were drastically improved by means of more efficient cell isolation methods, automation, and microfluidics techniques (Deng et al., 2014; Jaitin et al., 2014; Klein et al., 2015; Macosko et al., 2015; Paul et al., 2015; Zeisel et al., 2015), and noise was greatly reduced via implementation of unique molecular identifiers (Islam et al., 2014; Jaitin et al., 2014). Analytically, new algorithms for clustering, visualization, and modeling were developed uniquely for handling the sparse data measured by single-cell RNA sequencing (RNA-seq) (Haghverdi et al., 2015; Levine et al., 2015; Setty et al., 2016; Trapnell et al., 2014), while also controlling for and filtering various sources of technical noise produced during the single-cell profiling process (Brennecke et al., 2013; Haghverdi et al., 2015; Kharchenko et al., 2014; Levine et al., 2015; Paul et al., 2015; Setty et al., 2016; Trapnell et al., 2014; Wagner et al., 2016). It is now possible to isolate, measure, and model accurately and reproducibly gene expression profiles from hundreds of thousands of cells. Several excellent reviews provide more detailed information with regard to the recent analytical and experimental developments of single-cell RNA-seq technologies (Papalexi and Satija, 2017; Stegle et al., 2015; Tanay and Regev, 2017). In parallel, significant improvements were recently introduced into flow and mass cytometry (CyTOF) (Saeys et al., 2016; Spitzer et al., 2015), and these are rapidly synergizing with singl-cell genomic technologies, enabling researchers to focus on relevant cell populations and extending the single-cell RNA-seq findings to larger cohorts of cells, samples, and patients (Lavin et al., 2017). This massive expansion of scope calls for new and holistic strategies for the characterization of complex tissue-level immune processes in both

physiology and pathological states (Grün et al., 2015; Gury-BenAri et al., 2016; Habib et al., 2016; Jaitin et al., 2014; Matcovitch-Natan et al., 2016; Paul et al., 2015; Treutlein et al., 2014; Zeisel et al., 2015).

Lessons Learned so Far

The inherent functional diversity of the immune system has driven the community to develop tools for purification and analysis, which put immunology at the forefront of single-cell research from its very beginning (Fulwyler, 1965; Germain et al., 2006; Göhde and Dittrich, 1971). The immunology community now also greatly benefits from being early adopters and developers of single-cell RNA-seq for basic and biomedical immunology research. The first immune single-cell genomic studies focused on lymphoid organs such as the spleen, bone marrow, and thymus (Björklund et al., 2016; Brennecke et al., 2015; Gury-BenAri et al., 2016; Jaitin et al., 2014; Meredith et al., 2015; Paul et al., 2015; Schlitzer et al., 2015; Zhou et al., 2016). Scrutinized under this newly established molecular microscope, immune cell types so far considered homogenous were shown to display functional heterogeneity, even within thoroughly studied models and well-recognized immune cell populations. Recent studies demonstrated that classically defined immune entities can consist of different cell populations sharing overlapping markers (Björklund et al., 2016; Gaublomme et al., 2015; Gury-BenAri et al., 2016; Jaitin et al., 2014; Keren-Shaul et al., 2017; Paul et al., 2015; Schlitzer et al., 2015). For example, single-cell profiling of hematopoietic progenitor populations revealed a large degree of functional heterogeneity and commitment to various lineages (Paul et al., 2015; Schlitzer et al., 2015). Similarly, single-cell analysis of innate lymphocytes (ILCs) from mouse intestine and human tonsils uncovered heterogeneity exceeding the accepted separation into ILC1-3, as well as plasticity within these subsets (Björklund et al., 2016; Gury-BenAri et al., 2016). Furthermore, characterization of Th17 cells revealed heterogeneity and transcription programs involved in pathogenicity or regulatory potentials (Gaublomme et al., 2015). The discovery of disease-associated microglia (DAM), molecularly distinct microglia cells that interact with and phagocytize plaques in Alzheimer's disease, became possible due to the power of single-cell analysis to discover otherwise hidden and rare subsets of cells within immune populations (Keren-Shaul et al., 2017). Further elucidating the pathways and checkpoints of this new cell type may have important implications for future treatment of Alzheimer's disease and other neurodegenerative disorders. Another important generalization that stems from recent discoveries is that current cell classifications are not rigid or uniform, and upon challenges, almost all populations activate a heterogeneous response from seemingly similar cells types (such as the transition from naive to different T helper subsets) (Avraham et al., 2015a; Lönnberg et al., 2017; Shalek et al., 2013, 2014; Zhu et al., 2010). These results highlight the importance of heterogeneity in shaping and maintaining biological diversity, which reflects doubly on immune function, where extreme diversity (exemplified by the combinatorial assortment of VDJ recombination) must converge into robust and balanced macroscopic behaviors (Satija and Shalek, 2014). Clearly, as we sample less-characterized models and large patient cohorts, many yet-uncharacterized immune cells and pathways will emerge and highlight new hypotheses, therapeutic targets, and research directions.

Transcriptional Sorting: From Unbiased Sampling to Relevant Cell Types

Single-cell genomics hold the promise for answering many of immunology's most fundamental questions. These include the immune composition in various physiological and pathological conditions, such as response to infections, tissue development, neuronal maintenance and neurodegenerative diseases, metabolic regulation (Lackey and Olefsky, 2016), and the tumor microenvironment (Tirosh et al., 2016). It will also allow us to better understand the unique molecular pathways at play in each condition (Keren-Shaul et al., 2017). To start tackling the complexity of the immune system using single-cell genomics, we must devise a new methodology—reflecting on both experimental design and computational analysis. Careful attention and rigor must be applied to the preservation of the *in situ* cell states and original cell composition through optimization of tissue homogenization and/or fixation. In many cases, current tissue homogenization protocols are optimized for bulk methods or protein labeling, and careful calibrations are needed to maximize extraction of the different cell types while avoiding artificial induction of stress-related genes. A well-designed single-cell genomic experiment should exploit the advantages of the technology and *de novo* probe the entire spectrum of transcriptional states constituting the complex behavior of the immune system. In contrast to marker-based methodologies that seek purity and perfect separation, single-cell experiments should be as inclusive as possible—allowing reconstruction of immune populations involved in the process from the bottom up.

When planning a new study in animal models or humans, it is generally advised to start by sampling a few thousand cells in order to robustly reconstruct the main immune identities. Subtler distinctions between less abundant subtypes, such as between various monocyte states, require a much deeper or more focused design. There are two main approaches for measuring single-cell transcriptomes: full transcriptome coverage (Picelli et al., 2014; Ramsköld et al., 2012) and molecular counting methods that are based on sequencing of the 5' or 3' end of the molecule (Hashimshony et al., 2012; Jaitin et al., 2014; Macosko et al., 2015). Full coverage approaches are optimal for integrating mutations and/or inferring splicing events in single cells (Shalek et al., 2013), whereas counting methods are suited for cost effective single-cell RNA-seq profiling (Jaitin et al., 2014; Macosko et al., 2015). As a rule of thumb, in order to characterize a heterogeneous population composed of 20 transcriptionally distinct and evenly distributed cell states, 1,000–2,000 single cells (sampling ~50–100 cells per state, and allocating ~50,000 reads per cell) should be statistically sufficient for *de novo* clustering that can interrogate deeply the molecular profile of the different cell states (Heimberg et al., 2016; Tanay and Regev, 2017). However, in most cases, immune cells span several orders of magnitude in abundance, and for rare cells (< 1%), the marginal value in collecting more cells is diminished. Instead, a sequential approach should be considered, where tissue composition is first assessed in "low-magnification" by

sequencing 5,000–10,000 cells, while rare sub populations are probed more deeply by data-driven sorting strategies that include depletions and/or enrichment strategies based on known and new markers that are identified in an iterative fashion (Grün et al., 2015; Paul et al., 2015).

Exploiting the Power of Single-Cell Genomics in Immunology Research

One can start to imagine the new possibilities underlying single-cell RNA-seq research by picturing a holistic experimental design interrogating a complex, interconnected ecosystem, such as profiling the mutual influence of a tumor and the interacting immune and stromal cells (Figure 1A). The application of single-cell analysis in studying pathology is especially important, as pathology is often non-uniformly distributed in the tissue; for example, in liver or lung fibrosis, fibrotic and non-fibrotic areas will have different cell compositions. Thus, populations of interest tend to divide into pathological and naive states, a distinction often overlooked by bulk analysis relying on familiar FACS markers. The first step in successful profiling would be to *de novo* categorize the key cell types that are involved in the process by performing comprehensive profiling of the tumor and immune cells. This would be achieved by collecting and sequencing thousands of single tumor and immune cells, sampling the functional states within the tumor microenvironment—preferably from involved and uninvolved regions, as well as before and after immunotherapy treatment (Pardoll, 2012; Tirosh et al., 2016). Concurrent FACS recording of protein levels of lineage surface markers of each sorted cell (index sorting [Nestorowa et al., 2016; Paul et al., 2015; Velten et al., 2017]) would allow aligning the involved populations against previously annotated immune cell types and assessing the purity of these populations (Figure 1B). Additionally, extraction of tightly correlated gene modules across the data would reveal how specific pathways and cellular functions (e.g., proliferation, antigen presentation, exhaustion, differentiation, etc.) are distributed across cell types. Analysis of ligand-receptor pairs can suggest potential cross-talks within and between the immune and tumor cell states. Once novel (possibly rare) populations of interest are identified, their transcriptomes could be scanned for differentially expressed surface marker genes, which in the general case accurately represent differentially expressed protein markers (Keren-Shaul et al., 2017; Paul et al., 2015; Gury-BenAri et al., 2016). These can lead to development of new surface marker panels for negative and positive selection to efficiently sample and focus on relevant immune populations and deplete abundant and less relevant types. Similarly, panels of differentially expressed genes can be derived for CyTOF, allowing subsequent cost effective analysis of millions of cells (Gury-BenAri et al., 2016; Lavin et al., 2017). Imaging techniques such as immuno-histochemistry and smFISH can be used to highlight the spatial cellular organization and co-localization of differential genes in a tissue context (e.g., exhausted T cells in specific tumor niches) (Halpern et al., 2017; Keren-Shaul et al., 2017; Shah et al., 2016; Medaglia et al., 2017). Such data-driven approaches allow for efficient sampling of large patient cohorts for novel involved cell types and pathways, leading to *in vivo* and *in vitro* functional characterization experiments (Figure 1B).

Challenges and Future Developments

Alongside great promise, single-cell genomics technologies currently have several shortcomings that need consideration. A major analytical challenge is coping with large datasets of unbiased sampled cells, encompassing a large variety of cell types and cell states with various modular gene programs. This analysis requires the successful assignment of each cell type into accurate classifications, including subtle distinctions within cell types (for example, various T helper subsets or different dendritic cell types), while allowing the characterization of shared modular transcriptional programs (such as proliferation, inflammation, MHC-II pathway, exhaustion, or anti-viral response). Recent analytics have begun to cope with this complex challenge, and further advances will enable a detailed immune "table of elements" including all immune types and states that can be used as a reference (Butler and Satija, 2017; Keren-Shaul et al., 2017; Kharchenko et al., 2014; Paul et al., 2015). Importantly, following tissue homogenization, single-cell RNA-seq maps lack vital contextual information regarding space, clonality, time, metabolic processes, and epigenetic state. Current single-cell genomic profiling represents a transcriptional snapshot of dynamic and interacting networks of cells in different niches, which may overlook temporal and other processes that cannot be easily examined (Hoppe et al., 2014).

Nevertheless, with the flexibility of the technology and the rapid pace of innovation in the field, emerging next-generation adaptations of single-cell genomic methods are poised to make a dramatic leap forward by simultaneously recording and integrating complementary types of cellular and molecular information from the same cell (Adamson et al., 2016; Dey et al., 2015; Dixit et al., 2016; Jaitin et al., 2016) (Figure 2). Addition of sophisticated barcoded transcribed RNA molecules—interpretable by single-cell RNA-seq—enables the simultaneous recording of a cell's genetic perturbation and the resulting gene expression phenotype (Adamson et al., 2016; Amit et al., 2011; Dixit et al., 2016; Jaitin et al., 2016). Similar methods can be devised to encode and read additional information, such as cellular clonality via lineage barcoding (Junker et al., 2016; McKenna et al., 2016; Naik et al., 2013; Perié et al., 2015; Sun et al., 2014), the recording of past events by pulse-chase, inducible lineage tracing based methodologies (Bain et al., 2016; Perli et al., 2016), the simultaneous capture of chromatin state, and TCR or BCR sequences (Buenrostro et al., 2015; Cusanovich et al., 2015; DeKosky et al., 2013; Cheow et al., 2016; Han et al., 2014; Mooijman et al., 2016; Nagano et al., 2013; Smallwood et al., 2014). Recent studies used externally acquired information about expression of landmark genes in a spatial pattern to orient single cells on a differentiation axis or across a spatial gradient (Achim et al., 2015; Halpern et al., 2017; Satija et al., 2015). Alternatively, a different study implemented a technology that combines a photoactivatable fluorescent reporter with single-cell RNA-seq (NICHE-seq) to characterize the cellular organization of immune niches (Medaglia et al., 2017). FACS indices can further complement the transcriptional state with protein expression, as well as different fluorescent and genetic markers. Current approaches are mostly focused on extracellular markers, but development of improved fixation protocols will allow to combine the signaling activity of multiple pathways in parallel to the cellular state.

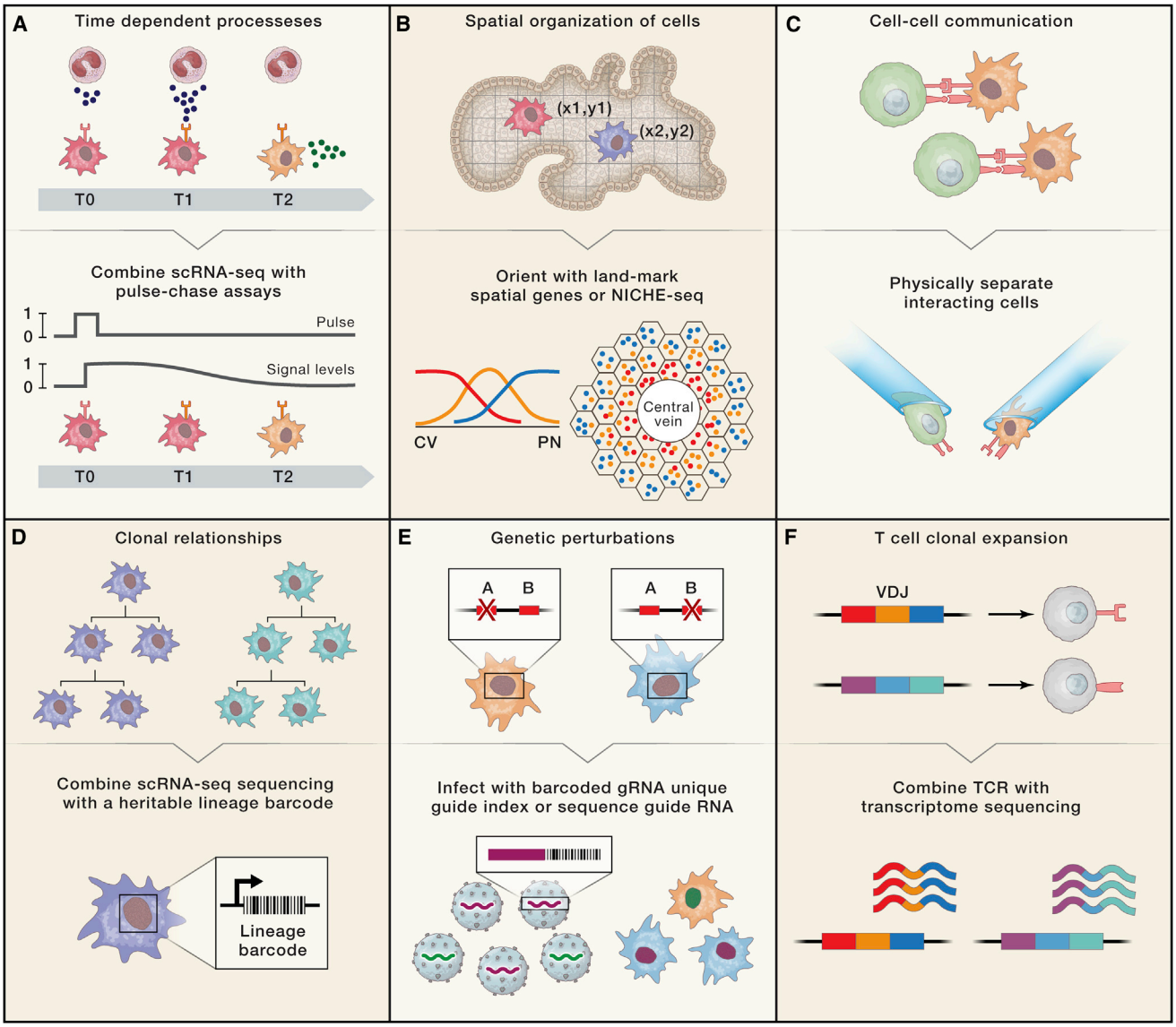

Figure 2. Novel Single-Cell Technologies for Integrating Complementary Cellular Information

Recently published and hypothesized approaches to solving important questions in immunology by integrating additional molecular, contextual, and temporal information with single-cell RNA-seq. Combining index sorting with pulse-chase and lineage-tracing experiments can provide temporal resolution to studies of dynamic processes (A); cell orientation by expression of landmark genes was shown to provide spatial resolution in single hepatocytes (Halpern et al., 2017), and use of photoactivatable mice were used to decipher cellular niches in lymphoid organs (NICHE-Seq; Medaglia et al., 2017) (B); physically separating and sequencing interacting cells can reveal immune crosstalks in resolution and scope never probed before (C); combining single-cell RNA-seq with molecular barcoding can be used to infer clonal relationships between cells (D) and for single-cell CRIPSR pooled screens to study the effects of genetic perturbations on immune function in different niches in single-cell resolution (Adamson et al., 2016; Dixit et al., 2016; Jaitin et al., 2016) (E); directed sequencing of the TCR/BCR region with the transcriptome of the same cell can correlate T/B cell clones with gene expression and regulation (Han et al., 2014) (F).

Combining these new exciting technologies will eventually enable to study not only single cells as isolated entities, but as dynamic and responsive elements inside a network of interacting cells. Specific attention should be paid to the molecular components allowing such interactions. Several new papers have demonstrated the potential of mapping pairs of expressed ligands to their corresponding receptors to allow the inference of the interactions between different cell types (Camp et al., 2017; Zepp et al., 2017). This approach is applicable to the immune system and can help to better understand known cross talks (such as between T and dendritic cells or thymic epithelial cells), as well as uncover unexpected connections and communication signals that facilitate immune activation or regulation in different models.

Reflecting back on the great endeavor of immunology to catalog all the immune cells, we are now better equipped to refine the central categories of "cell types" and "cell states." These definitions can be confusing at times, as immune cells

are endowed with fantastic plasticity and functional diversity. Lineage markers thought to represent a rigid identity fluctuate through time, are expressed differently following a challenge, or are found in different cell types and functional states at different tissue contexts (Vremec and Shortman, 1997). Further sub-cell-type classifications (e.g., Th1, Th2, Th17, Treg, etc.), while important, are prone to drastic inflation with addition of new markers and may overlook the true functional complexity of the immune system (Hume, 2008). Ultimately, data-driven approaches will likely refine our concepts by allowing researchers to define large assemblies of genes associated with all possible cell types and states in different chronological ages, as well as physiological and pathological niches. This will eventually lead to more relaxed models of immune identity, where different cells may acquire and lose overlapping expression programs depending on their location or stimulation.

Translational Immunology

Notwithstanding the noteworthy present achievements of single-cell genomics, its full potential to revolutionize immunology and immunotherapy has yet to be fully realized. Lowering costs and constant improvements in single-cell genomic technologies, combined with industrial and academic efforts to make single-cell genomics tools standardized and accessible to academia, the biotech industry, and the clinic, will soon turn single-cell genomics into a commonplace device for basic and applied immunology research. New computational tools are being devised in parallel for the standardization, accumulation, storage, and accessibility of single-cell data, the comparison of current and future datasets, and controlling for the immense variation of the immune system between individuals (Brodin et al., 2015). We envision that in the near future, large patient cohorts of various immune related pathologies (e.g., cancer, leukemia, neurodegeneration, metabolic disease, and autoimmunity) will be profiled with this new molecular microscope (Keren-Shaul et al., 2017; Tirosh et al., 2016), allowing for improved patient stratifications, identification of novel biomarkers, more relevant animal models, prediction of drug responses, and identification of novel cell targets and pathways, leading to new and more precise immunotherapy that is less harmful and more efficient.

ACKNOWLEDGMENTS

I.A. is supported by the Chan Zuckerberg Initiative; the Howard Hughes Medical Institute International Scholar Award; European Research Council Consolidator Grant 724471-HemTree2.0; a Melanoma Research Alliance Established Investigator Award (509044); the Israel Science Foundation (703/15); the Ernest and Bonnie Beutler Research Program of Excellence in Genomic Medicine; the Helen and Martin Kimmel Award for Innovative Investigation; a Minerva Stiftung research grant; the Israeli Ministry of Science, Technology, and Space; the David and Fela Shapell Family Foundation; the NeuroMac DFG (German Research Foundation)/Transregional Collaborative Research Center Grant; and the Abramson Family Center for Young Scientists. The authors declare patent applications relating to several parts of this Perspective.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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