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Review

Systems biology approaches to unravel lymphocyte subsets and function

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Single-cell technologies have revealed the extensive heterogeneity and complexity of the immune system. Systems biology approaches in immunology have taken advantage of the high-parameter, high-throughput data and analyzed immune cell types in a 'bottom-up' data-driven method. This approach has discovered previously unrecognized cell types and functions. Especially for human immunology, in which experimental manipulations are challenging, systems approach has become a successful means to investigate physiologically relevant contexts. This review focuses on the recent findings in lymphocyte biology, from their development, differentiation into subsets, and heterogeneity in their functions, enabled by these systems approaches. Furthermore, we review examples of the application of findings from systems approach studies and discuss how now to leave the rich dataset in the curse of high dimensionality.

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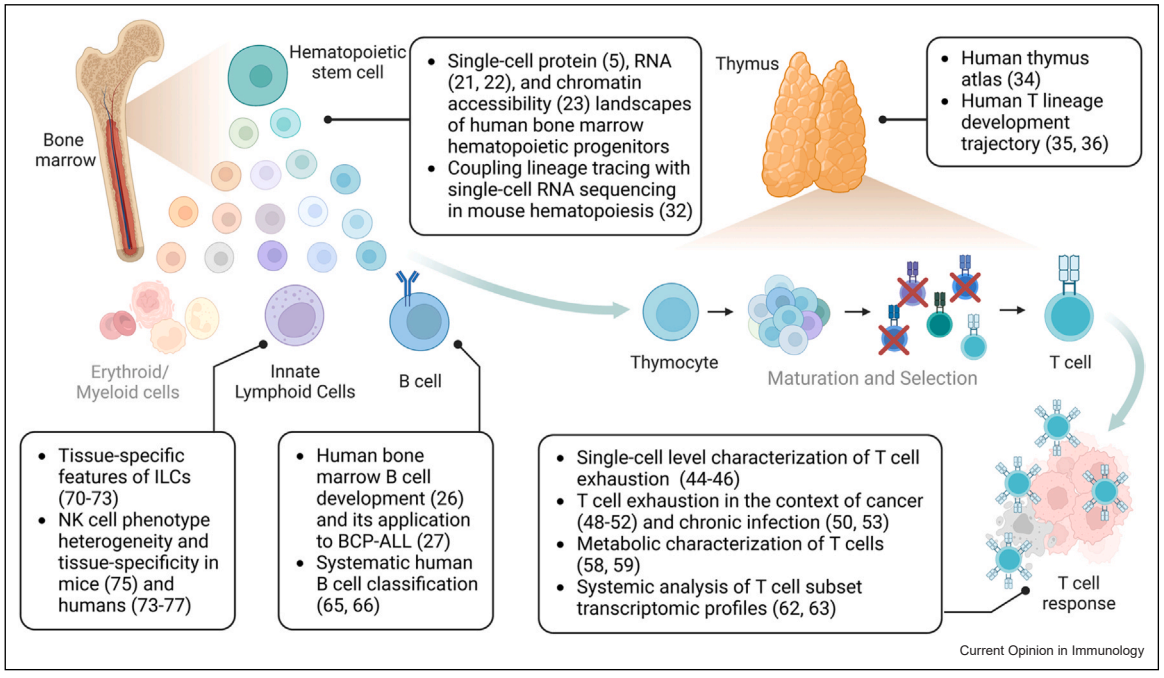
Introduction

The immune system is comprised of diverse sets of cells with distinct yet versatile functions that interact with each other to exert sophisticated immune responses. For example, the powerful effect of vaccines we are witnessing against COVID is mediated by B cells that secrete antigen-specific antibodies, but the effective production of antibody production requires activated CD4+ helper T cells. CD4 T-helper cells, on the other hand, require antigen-presenting cells, such as dendritic cells. Depending on how they were stimulated, CD4 T cells differentiate into different subsets with distinct cytokine profiles, which shape various immune responses. What we call an immune response at an organismal level is actually this intricate coordination of all these diverse cell types. Thus, to comprehend physiologically relevant immune responses, it is critical to understand the diversity of immune cells in the act and their interactions with each other. Systems immunology is the attempt to understand the immune system as a whole and has successfully broadened the spectrum of immunological understanding [1]. One type of the most popular tools in systems immunology is single-cell analysis, the earliest form of which was flow cytometry. In the hematopoietic immune systems, cells are conventionally defined by their immunophenotype (i.e. CD3 as a T-cell marker, CD19 as a B-cell marker). At the same time, there are functional states assigned via their effector function (i.e. antigen presentation, phagocytosis, and cytokine production). To capture both the diversity of cell types and states in a complex immune system, there is an inextricable link between single-cell techniques and the biological insights they can enable. Here, we review recent findings on lymphocyte differentiation, subsets, and functions by these systems approaches with single-cell technologies (Figure 1).

Systems analysis with single-cell technologies

The most familiar and established single-cell technique immunologists have is the fluorescence-activated cell sorter (FACS) [2]. The concept of labeling cells with fluorescent-tagged monoclonal antibodies and then analyzing them one at a time in the flow cytometer was first described by Leonard Herzenberg in 1969 [2,3]. Furthermore, the prospective isolation (or 'sorting') functionality of FACS enables downstream functional experiments with the exact cells identified by their flow cytometric properties. Ever since then, immunologists have identified, prospectively isolated, and analyzed the functions of immune cell types that match with a specific immune phenotype or master regulator transcription factor (i.e. CD25+ FoxP3+ regulatory T cells). Considering the widespread usage of this powerful tool in the history of immunology research, it is not an overstatement

Figure 1



Summary of lymphoid subsets and functions reviewed in this article. This article reviews the recent systems approach studies on lymphopoiesis in bone marrow and thymus and cell subtypes and functions of ILC, B cell, and T cell.

to say that modern immunology is based on the flow cytometry. Still, the limitation in the parameterization of these experiments (i.e. multiplexing) meant that most applications were still 'top-down', where cell populations of interest and their compositions were predetermined before the experiment.

Single-cell mass cytometry, also known as Cytometry by Time-of-flight (CyTOF), is a direct successor of flow cytometry with metal-isotope-tagged antibodies [4,5], instead of fluorescence-tagged antibodies. By measuring the metal-isotope-specific mass-to-charge ratio (m/z), mass cytometry increases the number of parameters measured by the cell to over 50 per cell on millions of cells per experiment [5,6]. Inheriting many aspects of flow cytometry (sample processing, staining, similar machine operations, data structure format, etc.), the increased number of parameters enabled a smooth transition of flow cytometry users into a systems approach with this tool. The technical advantages of mass cytometry and comparison with flow cytometry have been described well in other reviews [6,7]. Overall, this increased parameterization facilitates a more 'bottom-up', data-driven understanding of each experiment where previously unanticipated cell states and populations could be discovered and organized through unanticipated combinations of the molecular features measured.

With higher parameterization, but also higher cost-per-cell and therefore relatively lower cell number, single-cell sequencing has become more common and accessible to all fields of biology. Ever since its first appearance in 2009 [8], we have experienced a burst of different methods and throughput in single-cell RNA sequencing (scRNA-seq) in the last decade [9]. The recent publication of Tabula Sapiens [10] with nearly 500 000 cells from 24 tissues and organs, and similarly Tabula Muris a few years earlier [11], highlights the widespread application of scRNA-seq in different tissues as well as the abundance of public resources that can be utilized in a systems approach. Following the widespread impact of scRNA-seq in immunology research [12–14], technologies to analyze other modalities, such as chromatin accessibility [15,16], histone modifications [17], chromatin conformation [18], and so on, have also become available as high-throughput single-cell assays. More recently, new methods that combine measurements of multiple modalities, including RNA, protein epitopes, chromatin accessibility, spatial information, and more, have developed rapidly. Multimodal technologies and spatial information provide novel opportunities to study complex regulatory mechanisms and cell–cell interactions, as reviewed elsewhere [19,20]. For the scope of this article, we will focus on the biological findings on lymphocytes made with CyTOF and single-cell sequencing methods.

Single-cell techniques highlight the continuum of adult human hematopoiesis

The bottom-up, data-driven interpretation of next-generation single-cell datasets, has enabled researchers to investigate systems that are traditionally difficult to experimentally manipulate, such as human hematopoiesis bone marrow cells. Such approaches now allow the construction of dynamic models of cellular differentiation processes from an otherwise static sample because virtually all cell types and states are present at any one time and can be organized *in silico*. While the concept of hematopoiesis and hematopoietic stem and progenitors has been defined both in mice and humans, human bone marrow is not nearly accessible to intervene or experiment in its native condition compared with mouse models. Hence, with the advances in single-cell technologies, multiple groups have investigated human bone marrow hematopoiesis and demonstrated the molecular phenotypic continuum of hematopoietic progenitors in protein [5], RNA [21,22], and chromatin accessibility [23] landscapes. Such collection of high-throughput and high-dimensional data that span the whole developmental trajectory provided opportunities for the development of trajectory inference (TI) algorithms — often referred to as pseudotime analysis. In these computational techniques, cells are ordered by similarity in high-dimensional (RNA, protein, chromatin accessibility, etc.) space, with the aim of recapitulating the natural differentiation process in vivo [24]. Among multiple TI algorithms developed recently [24,25], one of the early exemplary uses of TI was a study with B lymphopoiesis in human bone marrow [26]. In this study, the authors developed a TI algorithm, Wanderlust, to recreate the B-lymphopoiesis pseudotime based on a single-cell mass cytometry analysis of bone marrow progenitors. Across pseudotime, there were specific coordination points at which multiple developmentally crucial events, such as the IL-7/STAT5 checkpoint, lead to the immunoglobulin gene rearrangement event. Moreover, the developmental trajectory of human B lymphopoiesis established in this paper became the basis for analyzing disease samples in another study with B-cell precursor acute lymphoblastic leukemia (ALL) [27]. Good et al. took a machine learning approach to assign B-leukemic blasts to the closest normal B-cell developmental population and identified developmentally dependent predictors of relapse that significantly enhance the risk prediction at diagnosis in ALL [27]. Similar approaches of utilizing normal single-cell hematopoietic data to analyze malignant leukemic samples have been used with RNA expression or chromatin accessibility data as well [28,29].

Along with the single-cell RNA-seq and TIs, novel methods for lineage tracing have evolved rapidly over the last decade [30]. Especially the combination of single-cell RNA-seq as a readout of lineage barcodes empowered scientists to compare the ground- truth trajectory by the lineage barcode to the trajectory inferred from the transcriptome of single cells [31]. Applying this method in hematopoiesis, Weinreb et al. revealed cell fate biases are made clonally early in differentiation, and also that clonally distinct hematopoietic progenitors can converge into a similar transcriptome, such as the monocyte development in the mouse steady-state hematopoiesis [32]. These methods and findings signify how the systems' approaches using single-cell methods are evolving to expand our knowledge.

T lymphopoiesis in human thymus examined by advanced tools

Another human hematopoietic organ that has been largely inaccessible for experimental manipulation is the thymus, in which later T-cell development takes place. While the complex steps of thymopoiesis have been studied extensively owing to mouse models [33], the translation of paradigms to human systems has been challenging, in part due to the scarcity of human thymus samples. Thus, recent scRNA-seq studies of human thymocytes [34–36] have considerably filled the knowledge gap in the field. By assaying single-cell transcriptome from more than 250 000 prenatal and postnatal thymic cells, Park et al. established a framework of human thymus development. Among 50 different cell states identified in the human thymus, including fibroblasts and epithelial cells, existed unconventional T cells were first reported in human thymus, such as CD8αα^{+} T cells, Natural Killer T (NKT)-like cells, and T_H17-like cells [34]. Another approach used by Le et al. and Lavaert et al. was to enrich the most premature precursors, earliest thymic progenitors (ETPs), by enriching for CD34+ cells in the thymus via FACS to study T-lineage developmental progression. Both groups utilized high-dimensional scRNA-seq data to define the developmental states of thymocytes and showed that the overall trajectory and expression of core regulatory genes are conserved between mouse and human thymopoiesis [35–37]. In particular, both studies identified a progenitor population expressing the Interlukin-3 (IL-3) receptor subunit CD123 that is transcriptionally primed for plasmacytoid dendritic cells in the thymus CD34+ compartment [35,36]. One remaining question is how these putative ETPs are connected to other cells in the hematopoietic hierarchy. While the fetal liver lymphomyeloid progenitors [38] seemed to bear the closest transcriptomic profile compared with the fetal thymic ETPs [34], the bone marrow counterpart remains ambiguous.

T-cell heterogeneities explained at the single-cell level

Even for T cells, likely the most-studied cell type in immunology, the heterogeneity across different tissue

sites and activation states has been a difficult topic to tackle. For example, T- cell exhaustion has been described for decades, but the term has been used broadly for a heterogeneous population of exhausted or dysfunctional T cells [39–41]. Alongside the seminal papers in the field that identified Thymocyte selection-associated high mobility group box factor (TOX) as the exhaustion marker via more traditional methods [42,43], numerous studies utilized scRNA-seq to analyze the heterogeneity of T-cell responses and discovered key gene regulation programs led by TOX [44] and T cell factor-1 (TCF-1) [45], driving these cell states. Especially the expression of TOX and the epigenetic landscape driven by TOX is demonstrated to be the crucial difference between other memory and effector T cells versus exhausted T cells [42–44,46,47]. The study of exhausted T cells using similar approaches has been extended to the context of chronic infection, cancer, and cancer immunotherapy with single-cell technologies [48–51] whose findings can be reviewed elsewhere [40,41]. Moreover, the VDJ sequencing of T cell receptors (TCRs) has enabled clonal lineage tracing of T cells in single-cell sequencing approaches. In the context of T-cell exhaustion, TCR sequencing was successfully applied in the basal cell carcinoma samples with checkpoint blockade to show novel clonotypes of T-cell expansion upon anti-Programmed cell death protein 1 (PD-1) treatment [52]. Another recent study with TCR sequencing showed that TCR signaling avidity correlated with different subsets of exhausted T cells in mouse Lymphocytic Choriomeningitis Virus (LCMV) models or human tumor-infiltrating lymphocytes [53].

Another crucial aspect of T cells that is frequently associated with their functional states is the cellular metabolism [54,55]. While the significance of metabolic regulation for different T- cell populations has been recognized previously [56,57], the bottom-up approaches to utilize the metabolic states of T cells to investigate their functional states at the single-cell level are fairly new. Recently, protein-based single-cell studies that specifically targeted the metabolic wiring of CD8 T cells reported metabolic states in accordance with functional states at the single-cell level [58,59]. These two studies utilized mass cytometry to quantify the protein expressions of regulators that served as surrogates to the overall activity of the respective pathway they resided in [58]. Specifically, Hartman et al. demonstrated that, more precisely than the broader expression of exhausted T-cell phenotypes CD39 and PD1, the metabolic- state indicators specifically corroborate the exhausted cell states, particularly within the tumor microenvironment [58]. Further discussions on T-cell immunometabolism can be found in other review articles [60,61].

To reinforce recent advances, researchers have proposed an updated T-cell atlas. Szabo et al., for instance, have described the tissue-specific signatures and CD4 or CD8 cell-type-specific activation states over 50 000 human T cells based on their transcriptomic profiles [62]. Orthogonally, Wang et al. have compared classical T-cell subsets by surface markers to T-cell populations defined by scRNA-seq profiles [63]. While these studies suggest more complex T-cell subtypes than conventionally defined, the authors conducted comparison with sorted populations to bridge the gap with what T-cell biologists currently use and what is observed in single-cell analyses. Moreover, the promise of data-driven bottom-up analysis of complex single-cell datasets is on full display where we are now able to differentiate all cellular T-cell diversity within an immune tissue in one analytical pass.

B-cell classification revisited with a systems approach

Compared with a plethora of T-cell studies with systems approaches, there have been limited attempts to systematically investigate the functional states of human B cells. In most cases, B-cell classification has been based on their maturation status, antibody repertoire, and antigen-experience states [64]. As an example, single-cell sequencing combined with VDJ sequencing was utilized to study the B-cell maturation trajectory during antibody class switching in tonsils [65]. Although antibody production is exclusively a B-cell-specific function, the other functions of B cells, such as antigen presentation and cytokine production, as well as tissue-specific B-cell subsets, should not be neglected. In this regard, a recent mass cytometry study screened for the expression of 351 surface molecules on human B cells in 4 lymphoid tissues (bone marrow, peripheral blood, tonsil, and lymph node) [66]. In this, Glass et al. identified 12 different populations of human B cells and interrogated their functions via subset-specific protein expression profiles. Among the 12 populations exists a $CD19^{hi}CD11c^{+}$ memory population that exhibited active metabolic and transcriptional state and a $CD39^{+}$ tonsil-resident population [66]. Moreover, the authors defined the gating schemes, CD45RB to the definition of early B-cell memory, for populations identified from their study. [66] Studies such as this, that connect high-dimensional multiomic datasets to 'human-interpretable' cell populations in low-dimensional space, provide a valuable resource for more focused studies to come.

Innate lymphoid cells: newest members of the family

Over the last decade and a half, immunologists continue to discover new Natural Killer (NK)-like lymphoid cell types (i.e. non-T and -B) that exhibit distinct cytokine production profiles [67,68]. They were soon given a uniform nomenclature as different groups of innate lymphoid cells (ILCs) based on the absence of

rearranged antigen receptors [69] and classified together with already well-known NK cells and lymphoid tissue inducer, due to their phenotypic similarities and common developmental progenitors [67]. As their critical roles in tissue homeostasis are emphasized, recent ILC studies highlight the tissue-specific transcriptomic programs that suggest tissue microenvironment-derived priming during ILC differentiation [70–73].

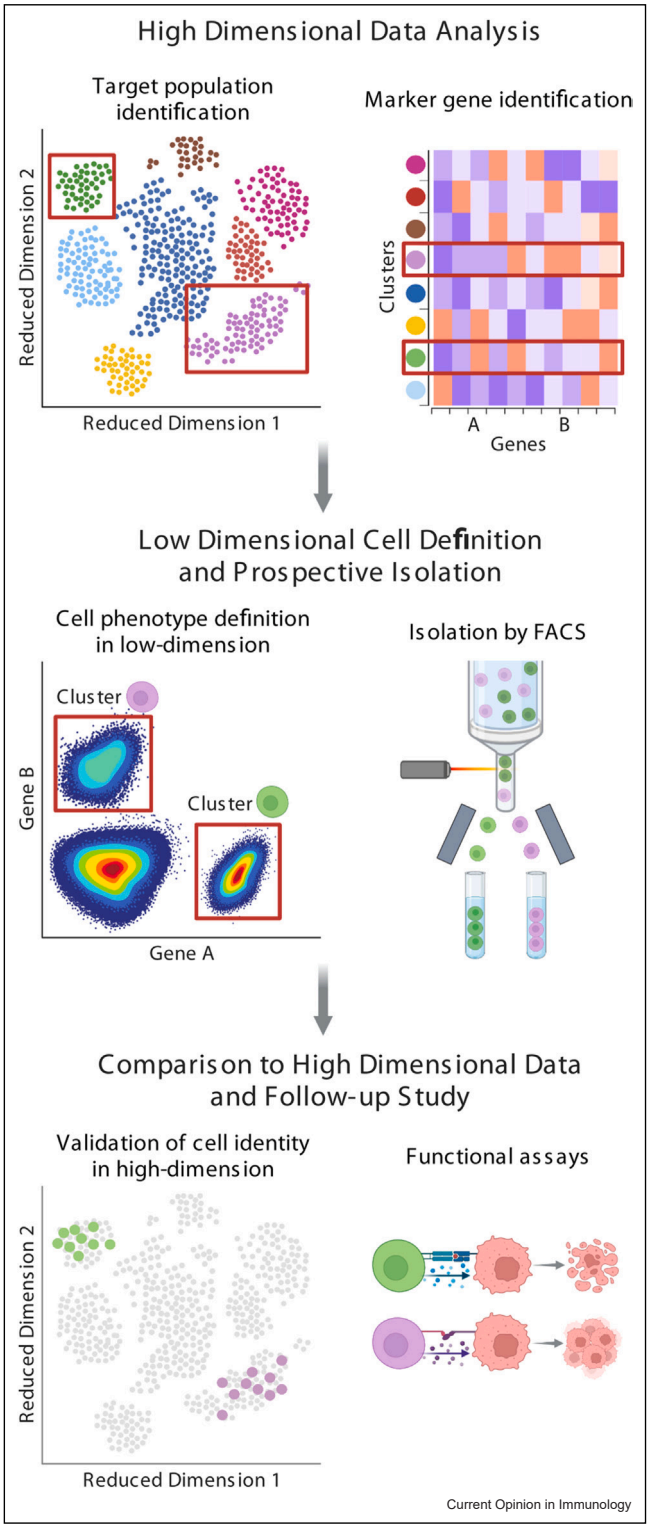
On the other hand, NK cells, the quintessential ILC, have historically been characterized as circulating cells in peripheral blood. Nonetheless, NK cells in peripheral blood already show a vast diversity of NK receptor expression phenotypes, as demonstrated in the single-cell mass cytometry study by Horowitz et al.. In this study, the authors concluded that the combinatorial expression of NK receptors could lead to an estimated 30 000 NK cell phenotypic populations in an individual [74]. Subsequently, various groups have utilized scRNA-seq to further describe NK cell diversity in mice and humans [75–77], in which tissue-specific NK subsets and differentiation trajectories were suggested. Considering their ability as a frontline defense against viruses and cancer, we anticipate a deeper understanding of NK cells to benefit the emerging NK cell-based immunotherapies [78,79].

Discussions — implications of immune heterogeneity

Single-cell studies have highlighted the immense diversity of cell types within the immune system where previously thought of as discrete cell populations contain new molecular phenotypes that are often continuously connected to one another. Still, systems approach studies rely on computational clustering algorithms to define populations, whose granularity can be set manually by a parameter. Hence, the old, but still valid, debate between 'lumpers and splitters' is back. How granular should one be in defining a cell type? Where is the bar between the plasticity of an immune cell and a distinct cell type? Single-cell studies without specific biological questions or functionally assayable endpoints could end up with broad but shallow analyses lacking meaningful insights.

To provide worthwhile knowledge to the field, a systems approach should be incorporated into the research as a means, not a goal. It is fundamental that studies on cell types and functional subsets need to be linked to specific definitions such that a 'novel' population can be the subject of follow-up studies. In any given modality, either a cell-type-specific phenotype or cell-type-specific signature matrix should be defined to minimize ambiguity and/or subjectivity and enable more mechanistic studies and validation of causal relationships (Figure 2). Researchers should also consider the implication of a

Figure 2



Example of high-dimensional data from a systems study translated into low-dimensional space and applied for follow-up studies. (Top) High-dimensional data from a systems approach are often analyzed with dimensional reduction algorithms or heatmaps. Based on this rich dataset, researchers often find a target population of interest (marked by

red boxes). It is critical to provide some type of signature phenotype for populations. (Middle) With the signature phenotype, the target population is now identifiable in the low-dimension analysis and can be prospectively isolated. (Bottom) Then, this population can be reanalyzed in the high-dimensional data to validate the cell identity and subjected to follow-up experiments and applications.

population in healthy and diseased context before claiming a new cell type to understand its role in homeostasis versus response to disruption.

Albeit complicated, a well-designed, purpose-driven systems approach can provide the most profound and transferable information in multiple layers. It is undeniable that the burst of new technological advances and the systems approach studies utilizing them are contributing to understanding the immune system more rapidly than ever.

Data Availability

No data were used for the research described in the article.

Conflict of interest statement

S.C.B. is a consultant and shareholders Ionpath Inc. that commercializes MIBI technology. S.C.B. is an inventor on, and receive royalties for patents relating to MIBI technology. W.J.G. is named as an inventor on patents describing ATAC-seq methods. 10X Genomics has licensed intellectual property on which W.J.G. is listed as an inventor. W.J.G. holds options in 10X Genomics and is a consultant for Ultima Genomics and Guardant Health. W.J.G. is a scientific co-founder of Protillion Biosciences.

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- •• of outstanding interest

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