Revolutionizing the Understanding of Immune Cell Heterogeneity through Single-Cell Transcriptomics

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Summary

Studying the immune system broadens our understanding of the underlying mechanisms contributing to health and disease. Recently, single-cell RNA sequencing (scRNA-seq) has provided an alternative unbiased approach to study gene expression on a single cell-level and has emerged as one of the leading techniques to uncover the immune system's complex networks. It has allowed researchers to discover rare cell types and novel cell states in the immune system and has provided a comprehensive overview of the novel insights in immune cell characteristics, temporal, and spatial distribution as well as cellular function. Here, a review summarizing the current state of research of single-cell transcriptomics is provided, focusing on hematopoietic origin, novel immune cell types in thymus organogenesis, as well as the developing and the adult immune system. Moreover, we highlight the limitations inherent to the scRNA-seq experimental pipeline that propose challenges in advancing our knowledge of the comprehensive immune cell landscape.

Abstract

The immune system is comprised of complex networks, cell types, signaling and interactions producing a highly functional cellular ecosystem to maintain immune homeostasis. Single-cell techniques, specifically single-cell RNA sequencing (scRNA-seq), have catalyzed a revolution to study the immune system's inherent complexity by providing an unbiased approach to characterize immune cell types, trajectories and signaling. Here, we review recent advances by scRNA-seq studies deconvolving the immune system's heterogeneity. We discuss how single cell transcriptomics has presented a novel perspective on hematopoietic origin, differentiation trajectories and lineage commitment. Furthermore, we highlight newly discovered immune cell types and alternative cellular functions in thymus organogenesis, as well as the prenatal and adult immune system. The article concludes by discussing the technical, computational, and biological limitations of scRNA-seq, and how these can be partially resolved by including multiple multi-omics techniques.

Introduction

The immune system is a host defense system composed of various highly specialized cell lineages that work together in a complex network of intracellular signaling and intercellular interactions to prevent, clear, and memorize pathogenic infections throughout an organism. These cells either reside in lymphoid and non-lymphoid tissue, or transit through the body via the vascular and lymphatic system. Lymphoid tissue includes primary lymphoid tissue such as the bone marrow (BM), fetal liver (FL) and thymus, which represent the sites of lymphocyte development, as well as secondary lymphoid tissue comprised of the lymph nodes, spleen, tonsils, and Peyer's patches, where mature cells reside (Thompson, 2012). Studying cell diversity allows insight into the development, differentiation, relation, and function of cells in the complex immune network.

Previously, the categorization of immune cells was established by 'targeted' single-cell techniques including immunofluorescence, fluorescence activated cell sorting (FACS) and flow cytometry, which are limited to probing a few selected proteins, thereby introducing bias to pre-determined genes (Bajénoff & Germain, 2007; Perfetto et al., 2004; Stubbington et al., 2017). 'bulk' RNA sequencing determinates the average transcription profile of a heterogenous cell mixture, which impedes studying cellular diversity. Although these previous technologies are limited in their application, they have contributed immensely to the discovery and characterization of many immunological cell types.

Recently, single-cell RNA sequencing (scRNA-seq) has rapidly evolved our ability to characterize the immune system's distinct molecular profiles by providing an unbiased, alternative technique that allows sequencing and categorizing cells without prior knowledge of genes

or proteins of interest. Based on transcriptome analysis rather than cell surface markers, scRNA-seq has the capacity to elucidate genomic, epigenomic, and transcriptomic heterogeneity, as well as spatial and temporal distribution on these levels (Chen et al., 2019; Kashima et al., 2019). The development of low input scRNA-seq protocols triggered an avalanche of studies discovering rare cell types and novel cell states in the immune system.

Here, we provide a comprehensive overview of the novel insights in immune cell characteristics, temporal, and spatial distribution as well as cellular function revealed by scRNA-seq. We explore the current state of research regarding hematopoietic hierarchy, the developmental and adult immune system, including newly emerged immunological cellular subsets. Furthermore, we determine the shortcomings of scRNA-seq and how these can be partially reduced by including other single cell techniques.

Disrupting the Classical Model of Hematopoiesis: A New Paradigm Revealed by Single-Cell Transcriptomics Studies

Hematopoiesis represents the production of blood and immune cells, which is a continuous process maintained and balanced throughout the lifetime of an individual. Hematopoietic stem cells (HSCs) harbor the capacity of self-renewal and multipotent differentiation, which led to the concept of a 'hematopoietic hierarchy'. This hierarchy explains hematopoietic differentiation through a set of bifurcations including multipotent, oligopotent, followed by unilineage progenitor stages coupled with decreasing self-renewal capacity and loss of lineage potential. This hierarchical classical model of hematopoiesis depicts a highly compartmentalized and stable structure, which has been challenged recently by scRNA-seq studies providing a complete and unbiased view of HSCs, progenitors, cellular relationships, and lineage differentiation routes.

This novel paradigm in hematopoiesis (depicted in Figure 1) describes lineage commitment as a continuous process, composed of hematopoietic stem and progenitor cells (HSPCs) that differentiate directly towards unilineage-restricted cells, without any major transitional stages (Velten et al., 2017). ScRNA-seq data revealed that HSCs, multi potent progenitors (MPPs) and multilymphoid progenitors (MLPs) were interconnected as a single fluid transcriptional 'cloud' and represent transitory states, which was supported by in vitro single-cell culture assays. The most immature HSPCs are characterized by quiescence and the discrete populations downstream of the HSPC continuum indicated distinct lineages of all major branches of BM hematopoiesis that do not pass through the traditional hierarchy of stable multi-, oligo- and unipotent progenitor populations (Velten et al., 2017). In line with these observations, multipotent cells in the adult BM solely populated the stem cell compartment, whereas the progenitor population was dominated by unilineage progenitors with very few oligopotent intermediates in human (Notta et al., 2016) and in mouse (Giladi et al., 2018; Tusi et al., 2018). Furthermore, lymphoid-primed multi-potential progenitors (LMPPs), granulocyte-macrophage progenitors (GMPs) and MLPs formed a transcriptional continuum, in which rare multi-lineage lympho-myeloid progenitors were observed, but unilineage progenitors were far more abundant (Karamitros et al., 2018).

Single-cell sequencing assay for transposase-accessible chromatin (scATAC-seq) reveals the accessibility of chromatin, which typically represents transcriptionally active genes. Variation in chromatin accessibility of transcription factor (TF) motifs indicated by scATAC-seq, displayed regulatory heterogeneity of the adult BM phenotypic MPP population, possibly indicating lineage priming (Buenrostro et al., 2018). A transcriptional map of the developing fetal hematopoietic system 7-17 post conception weeks (pcw) supported this notion by identifying differentiation towards lymphoid, myeloid, and megakaryocyte–erythroid–mast cell (MEM) progenitor populations within the HSC/MPP compartment (Popescu et al., 2019). In contrast to the previous study, Ranzoni et al proposed that lineage priming in the fetal HSC compartment 17-22 pcw does not occur on a transcriptional level, rather they observed differential chromatin accessibility and lineage specific TF activity before the onset of lineage commitment in the HSC/MPP population indicating epigenetic, but not transcriptional, lineage priming, as seen in adult BM (Buenrostro et al., 2018). Potentially, Ranzoni et al 's scRNA-seq dataset was too limited to capture the results of (Popescu et al., 2019), as their HSC/MPP compartment did sporadically express lineage-affiliated genes, but not consistently. Since multiple transcriptional states can be associated with a specific chromatin accessibility profile, the HSC/MPP transcriptome may be too heterogeneous to capture lineage priming in Ranzoni et al 's data.

Multiple studies also report a bias towards the megakaryocyte lineage in the immature HSC/MPP

continuum, indicating that megakaryocyte/erythroid lineage commitment may be one of the earliest fate decisions as divergence from the lymphoid/myeloid compartment occurs within the HSC/MPP population (Belluschi et al., 2018; Rodriguez-Fraticelli et al., 2018; Tusi et al., 2018). Conversely, the human prenatal hematopoietic system does harbor oligopotent intermediates with megakaryocyte/erythroid potential in the FL (Notta et al., 2016). In summary, single cell transcriptomics studies have proposed a novel paradigm for hematopoiesis, where multilineage progenitors undergoing lineage priming are restricted to the HSC/MPP compartment, and unilineage progenitors derive from the HSC continuum without transitional stages, highlighting a fluidic nature of differentiation.

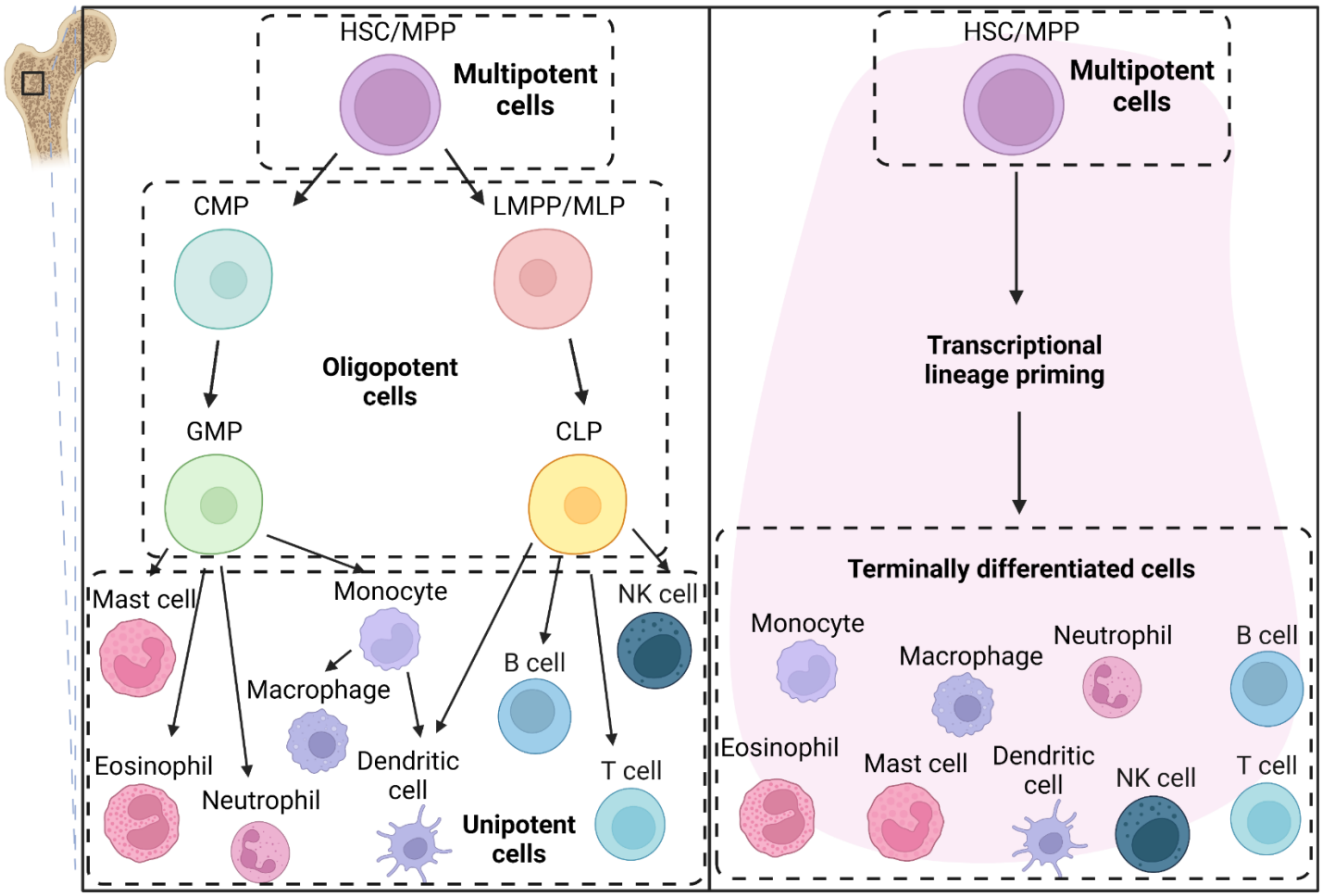

Figure 1: The Novel Paradigm in Hematopoietic Origin. The classical view of hematopoiesis (left) describes a hierarchical tree, where a set of bifurcations produce intermediate progenitor cellular states characterized by a loss of lineage potential. Recently, scRNAseq studies have proposed a HSC continuum (right), where HSCs harboring multilineage potential are transcriptionally primed for unilineage differentiation. In this model, the multi potent cells in the HSC/MPP compartment form a transcriptional cloud and the stable multi-, oligo- and unipotent progenitor populations are absent. Epigenetic and transcriptional priming in the HSC compartment determines the differentiation trajectory.

Insights from scRNA-seq Studies during Thymus Organogenesis are Uncovering T cell Development

Progenitor subsets

The thymus is a central organ in the establishment of adaptive immunity as it mediates the development of T cells. During human thymus organogenesis, thymic epithelial cells (TECs) differentiate and thymus seeding progenitors (TSPs) migrate from circulation towards the developing fetal thymus at gestational week 8 (Farley et al., 2013). These hematopoietic TSPs give rise to early T progenitors (ETPs), which are the most primitive hematopoietic cells in the developing thymus with T lymphocyte potential, followed by the selection, differentiation, and maturation of T cells. After week 12, mature $CD3^+CD4^+$ and $CD3^+CD8^+$ single positive (SP) T lymphocytes can be detected in the thymus. Until recently, the characterization of TSPs and the TSP to ETP developmental trajectory was poorly understood due to cell scarcity. Combined analysis of scRNA-seq datasets revealed a lymphoid progenitor subpopulation in the FL at Carnegie Stage (CS) 20 and CS23 with a similar transcriptional signature to ETPs, indicative of their TSP property (Zeng et al., 2019) and trajectory analysis revealed that TSPs differentiate to ETPs through an intermediate proliferating ETP population. A separate migration path of TSPs into the thymic primordium prior to and after thymic vascularization at week 9 was proposed before (Farley et al., 2013), and supported by the differential expression of cell migration and adhesion genes in embryonic ETPs (only present in week 8) and fetal ETPs (present after week 9) (Zeng et al., 2019). The pre-thymic lymphoid progenitors of these TSPs were detected as early as week 5 in the aorto-gonad-mesonephros (AGM) region, which represents the source of definitive hematopoiesis in human development 9-11 days postcoitum (dpc) until the principal site of hematopoiesis shifts to the FL at 5-8 pcw. This is particularly interesting, as it is in line with emerging evidence indicating that certain lineage progenitors emerge prior to, and possibly independent of conventional HSCs (Ghosn et al., 2019).

In the postnatal human stage, two distinct BM-hematopoietic progenitor derived TSP subsets with differential expression of CD7 and CD10 and distinct T and non-T lineage potential were identified (Lavaert et al., 2020). Thymic progenitor populations with non-T lineage potential predominantly give rise to plasmacytoid dendritic cells (pDCs), suggesting a specific differentiation pathway for pDCs in human development (Le et al., 2020). Similar to humans, murine ETP subsets were identified with varying multilineage potential in early murine development, suggesting multilineage priming in individual ETPs (Zhou et al., 2019). Contrastingly, the pDC primed thymic progenitor population was not observed in murine scRNA-seq datasets, which could be explained by the distinct species-related transcriptomes during the initial stages of thymopoiesis in humans and mice (Le et al., 2020). Thus, scRNA-seq studies have shed light on the origin and differentiation trajectory of hematopoietic cells during thymus organogenesis.

TCR rearrangement and Treg formation

T-lineage committed thymocytes undergo T cell receptor (TCR) rearrangement and then differentiate to $CD4^+CD8^+$ double positive (DP) cells, accompanied with CD4 and CD8 upregulation, followed by differentiation to $CD4^+$ or $CD8^+$ SP cells. The mechanisms of conventional $CD4^+$ and $CD8^+$ T cell differentiation are highly conserved in humans and mice (Chopp et al., 2020). TCR rearrangement drives TCR repertoire diversity, which is a fundamental characteristic of an effective immune system as it predisposes the ability to respond to peptide-MHC (pMHC) combinations. The rearrangement of TCR loci occurs in different T cell differentiation stages and although the TCR repertoire formation is stochastic, scRNA-seq revealed overlapping TCRβ sequences in individuals (Khosravi-Maharlooei et al., 2019). This is attributed to a bias in VDJ usage by gene locus position and thymus selection enriching these TCRβ sequences (Khosravi-Maharlooei et al., 2019; Park et al., 2020).

Regulatory T cells (Tregs) are a specialized $CD4^+$ T cell subset that suppress activated T cell subsets through a multitude of mechanisms to maintain immune homeostasis. ScRNA-seq studies have elucidated the previously poorly characterized Treg differentiation pathway. A scRNA-seq cell atlas of thymic development connected αβT cells to Tregs differentiation, supporting the notion that Tregs are derived from thymocytes with intermediate TCR antigen affinity (Park et al., 2020). Another study observed a large fraction of autoreactive TCRs in both Tregs and $CD4^+$ SP T cells using scTCRseq, confirming that the process of negative selection is inherently leaky and self-reactive TCRs that escape Treg differentiation are capable of giving rise to $CD4^+$ SP T cells (Owen et al., 2022).

scRNA-seq also revealed that the mature Treg cell repertoire is comprised of two distinct developmental programs, characterized by differential expression of

CD25 and FOXP3, affinity to self-antigens, TCR repertoire, transcriptomes, use of signaling pathways and selection program (Owen et al., 2019). For the $CD25^+$ Treg progenitor, a novel function in Treg maturation was proposed, as this progenitor population may be responsible for the IL-2 production driving the conversion of Treg progenitors to mature Tregs, thereby enabling their differentiation in an autocrine manner (Owen et al., 2022). Furthermore, resident thymic Tregs (RT-Tregs) have been observed before for several years but were recently characterized on a single cell transcriptional level in the same study (Owen et al., 2022). RT-Tregs display not only transcriptional heterogeneity, but also the highest degree of TCR diversity, which is likely involved in orchestrating Treg differentiation. Future scRNA-seq could further elucidate the cellular function of the RT-Treg subset. Thus, scRNA-seq and scTCRseq combined multi omics analyses have provided novel insights in TCR sequences and differentiation trajectories of CD4 and CD8 SP T cells and Tregs.

Unconventional T cells

γδ T cells, a class of unconventional T cells characterized by γδ TCR expression rather than αβ TCR expression, first branch from the initial DN ETP stage during embryonic development (Park et al., 2020; Pellicci et al., 2020; Zeng et al., 2019), indicating their development occurs earlier then αβ T cells. ScRNA-seq revealed that transcriptional programming of mature γδ T cells in the fetal thymus leads to three effector fates; Type 1 (secreting IFN-g), Type2-like (secreting IL-4) or Type 3 (secreting IL-17), with distinct developmental trajectories and abundance depending on the gestational stage (Narayan et al., 2012; Sanchez Sanchez et al., 2022). This contrasts with pediatric γδ effector T cells which represents a small population only programmed for Type 1 or Type 3 immunity (Sanchez Sanchez et al., 2022). Other prenatal unconventional T cells include TH17–like cells, Natural killer T (NKT)–like cells, and $CD8\alpha\alpha^+$ T cells. scTCRseq indicated these prenatal subsets express mostly but not only αβ TCR sequences, suggesting lineage convergence of some αβ and γδ T cells (Park et al., 2020). Prenatal unconventional T cells expressing an αβ TCR display a TCR rearrangement developmentally similar to DP T cells, supporting the hypothesis of thymocyte-thymocyte (T-T) origin where DP T cells mediate the positive selection signaling of neighboring DP T cells, rather than cTEC (Park et al., 2020). Since TH17-like and NKT-like cells are absent from the postnatal thymus, they are presumed to be developmentally specific, whereas $CD8\alpha\alpha^+$ T cells were present in the pediatric thymus but declined in adulthood (Suo et al., 2022).

In contrast to prenatal unconventional T cells, adult unconventional T cells expressing an αβ TCR such as invariant natural killer T cells (iNKT cells) and mucosal-associated invariant T cells (MAIT cells) display a TCR repertoire limited in diversity (Pellicci et al., 2020). scRNA-seq also revealed that γδ T cells, iNKT and MAIT cells mature via a shared effector differentiation pattern in the thymus, harboring analogous effector subsets for Type 1, Type 2 or Type 3 immunity, as described above for fetal γδ T cells (Lee et al., 2020). A scRNA-seq study proposed a model in which iNKT development emerges via iNKT2 as a lineage precursor, which can give rise to iNKT1 or iNKT17 sublineages (Baranek et al., 2020). Interestingly, this model aligns with the development and transcriptome of MAIT cells, as the iNKT2 transcriptional signature was similar to a transitional MAIT cluster giving rise to MAIT1 and MAIT17 cells (Baranek et al., 2020; Koay et al., 2019). Whether these iNKT2 and MAIT2 clusters represent intermediate or terminally differentiated clusters, is uncertain. Altogether, scRNA-seq has uncovered the developmental and differentiation trajectory of prenatal and adult unconventional T cells. However, many aspects of these relatively unexplored cell types remain elusive.

Thymic epithelial cells

Thymic epithelial cells (TECs), localized in the thymic cortex (cTECs) and medulla (mTECs), are critical for the development of self-tolerant T cells. cTECs mediate T cell lineage commitment and positive selection, whereas negative selection and Treg differentiation are orchestrated by mTECs. scRNA-seq revealed dynamic transcriptional changes in TEC heterogeneity during thymic and thymocyte development, across human lifespan (Park et al., 2020). Adult TECs upregulate MHC pathway and downregulate cell cycle genes compared to embryonic TECs (Bornstein et al., 2018). During early development at 7-8 pcw, cTECs are more dominant and proliferating then mTECs, consistent with their function as positive selection of T cells precedes negative selection (Park et al., 2020; Zeng et al., 2019). Four major mTEC populations are conserved in humans (Park et al., 2020) and mice (Bornstein et al., 2018), emerging in late embryonic development or after birth, including a novel rare mTEC population (mTEC IV) with transcriptional similarity to the gut chemosensory epithelial tuft cells. Loss of Tuft-like mTEC IV cells increases the thymic ILC2 population but does not affect the development of other TEC populations (Bornstein et

al., 2018). The function of Type 2 iNKT cells, which shape an IL-4 rich thymic medulla, is also mediated by these IL-25 producing Tuft-like mTEC IV cells, indicating they are involved in the development of multiple Type 2 immune cells (Miller et al., 2018). Taken together, scRNA-seq has reshaped our understanding of TEC cellular function, with alternative roles for TEC subsets in specific stages of life.

Uncovering the Heterogeneity and Developmental Trajectories of Prenatal Innate Immune Cells through scRNA-seq

ILCs

Innate lymphoid cells (ILCs) have similar phenotypes, function, and developmental trajectories as T cells, while lacking their expression of lineage markers and antigen receptors. Similar to iNKT and γδ T cells, ILCs are activated by environmental signals, making them capable of performing their effector function early in the immune response and playing a central role in regulation. Due to the characteristics defining ILCs, as well as their scarcity, capturing ILCs for research is a challenge. scRNA-seq resolves these issues and has indicated substantial heterogeneity in ILCs. ILCs are categorized in, previously three, but now five groups; Natural killer (NK) cells, ILC1, ILC2, ILC3 and lymphoid tissue-inducer (LTi) cells, of which ILC1-3 were only observed fetal thymus of human and mouse (Kernfeld et al., 2018; Zeng et al., 2019). ILCs likely share a lymphoid progenitor with T cells in the FL of mice and human, which bifurcates to ILC progenitor (ILCP) and LTi progenitor (LTiP) and commitment of ILC fate is characterized by loss of multilineage lymphoid potential (Ishizuka et al., 2016; Popescu et al., 2019). During early development, NK cells and ILCPs in non-lymphoid tissue (NLT) share a similar transcriptome to their liver counterparts, which diverges after tissue-related maturation (Popescu et al., 2019). ILCPs were more abundant in the intestine, then the fetal liver or other lymphoid tissue indicating a critical role for the intestine in the development of ILCs (Liu et al., 2021).

LTis, previously categorized in the ILC3 compartment, mediate the development of secondary lymphoid tissue, as well as mTECs maturation in the mouse embryonic and neonatal thymus, but are absent from the adult thymus. Contrastingly, ILC2s are present in the human fetal thymus, but the loss of LTis in adulthood enables ILC2 to become the dominant ILC population in the adult thymus (Jones et al., 2018; Zeng et al., 2019). ILC maturation occurs in the tissue, explaining the heterogeneity of ILCs from organ to organ, as site specific environmental cues lead to tissue-specific transcriptional imprinting (Mazzurana et al., 2021). Thus, scRNA-seq has shed light on the developmental pathways of various ILCs subsets, a lineage previously poorly characterized due to technical limitations.

Dendritic Cells

Dendritic cells (DCs) are the most potent antigen presenting cells (APCs) capable of activating naïve T cells and thereby playing a critical role in host immunity. Recently, scRNA-seq studies have indicated alternative function and origin of DCs during prenatal development. DC1, DC2 and Plasmacytoid DC (pDC), a rare immune cell type that produce massive amounts of type I interferons (IFN-I), branch from a mixed lymphoid and myeloid origin (Popescu et al., 2019; Tusi et al., 2018). Although DCs have been described to originate from HSCs, scRNA-seq revealed DC subclusters DC1 and DC2 were detectable in the FL prior to the establishment of the fetal BM, proposing an alternative role for DCs during development (Popescu et al., 2019). Fetal DCs may have a function in mediating tolerance to self and maternal antigens by promoting Treg induction and inhibiting T cell TNF-α production (McGovern et al., 2017).

Another proposed role of DCs during fetal development, describes the colocalization of DC1 with unconventional T cells in the thymic medulla while conventional T cells colocalized with mTEC, as revealed by spatial single cell transcriptomics (Suo et al., 2022). Possibly, DC1 mediates the negative selection of prenatal unconventional T cells in the thymic medulla, as mTECs do for conventional T cells. In fetal skin, DCs emerge approximately 7 pcw, and increase in abundance over the course of gestation as the fetal skin barrier is formed (Popescu et al., 2019; Xu et al., 2021). Besides these alternative functions of DCs during fetal development, DC subsets in adult and prenatal counterparts are highly conserved (Suo et al., 2022). In summary, scRNA-seq has proposed alternative functions for prenatal and adult DCs, which may be vital for the development of a functional immune system.

Macrophages

Macrophages are immune cells characterized by avid phagocytosis that reside in a variety of tissues throughout the body. Recent scRNA-seq findings suggest macrophages may have alternative developmental trajectories and prenatal functions as previously thought. Macrophages emerge as one of the first immune cells in fetal development, as they are

observed in the yolk sac (YS) as early as 6 pcw before the appearance of HSPCs (Bian et al., 2020; Popescu et al., 2019). Two waves, both prior and independent of HSPs appearance, comprise the emergence of YS-derived tissue-resident macrophages; an early population in the YS, which contributes to the main macrophage population, and a population of YS monocyte-derived macrophages. Embryonic tissue-resident macrophages that seed the peripheral tissue are long-lived but can be replaced by circulating blood monocytes (MacParland et al., 2018). While monocytes and DCs in NLT display transcriptional similarity with FL counterparts, the macrophage transcriptome is highly specific for its tissue residence, indicating macrophage maturation in the periphery. Since HSC-independent derived macrophages display a differential transcriptional signature from their HSC-derived counterparts, future studies should consider the macrophage origin when characterizing macrophage subsets in disease.

In early gestation, macrophages and mast cells display a proliferative transcriptome and an angiogenesis associated proinflammatory phenotype across tissues, suggesting of their role in prenatal angiogenesis and homeostasis prior to adapting their classical immune effector function 10-12 pcw (Suo et al., 2022). Since the lymphatic system develops parallel to the acquisition of these immune effector functions, future scRNA-seq studies could shed light on the mechanism underlying these dynamic transcriptional changes. Thus, single cell transcriptomics has elucidated alternative functions for macrophages during fetal development, highlighting the intricate regulatory landscape of fetal development.

Insights into Cellular Heterogeneity and Functions of the Adult Immune System via scRNA-seq Studies

T cells

Activated $CD8^+$ T cells differentiate to effector T cells (Teff) or memory T cells (Tmem) in acute infections, or exhausted T cells (Texh) in chronic infections and cancer. This process is facilitated by multiple cellular subsets, including other $CD4^+$ T cells, such as T helper 2 (Th2) cells and T helper 17 (Th17) cells, and other innate macrophages and DCs. The Texh cells can be further categorized in progenitor, intermediate and terminal cells, the latter characterized by high expression of inhibitory receptors. Studying these cellular subsets via scRNA-seq provides novel insights into underlying mechanisms preceding health and disease. Zanger and colleagues defined a novel T cell subset, $CX3CR1^+$ $CD8^+$ T cell, which displayed potent cytolytic capacity in chronic infection via KLR genes and was required for viral control (Zander et al., 2019). Furthermore, the $CX3CR1^+$ $CD8^+$ T cell subset formation was mediated by IL-21 producing $CD4^+$ T cells. Recently, a Texh subset expressing KLR genes, likely determined as intermediate Texh before, was revealed (Giles et al., 2022). The expression of cytolytic KLR genes is particularly interesting as it is reminiscent of the cytolytic $CD8^+$ population identified by Zander and colleagues (Zander et al., 2019). The NK like Texh cell represents a heterogenous population expressing $CX3CR1^+$ and KLR genes, capable of functional diversification and harboring more cytolytic potential than other Texh subsets (Szabo et al., 2019). Although $CD8^+$ T cells in acute infection may express NK receptors as well, the NK-like Texh population is transcriptionally distinct from Teff and Tmem cells, despite overlapping epigenetic and transcriptional circuits. Since exhaustion is a hallmark of TILs and chronic infection, further research in this novel KLR expressing Texh subset could benefit cancer immunotherapy.

Multiple scRNA-seq studies reported a novel rare $CD4^+$ T cell subset characterized by high expression of Interferon stimulated genes (ISGs) following immunological challenges (Kiner et al., 2021; Tibbitt et al., 2019). These ISG-$CD4^+$ T cells are absent from lymphoid tissue but induced upon an active immune reaction. Although the origin and function of the rare ISG-$CD4^+$ T cells has remained elusive, their high expression of ISGs and emergence upon immune challenges suggests their participation in immune pathogenesis (Zemmour et al., 2020). Furthermore, other scRNA-seq studies report a continuous trajectory of T cell subset heterogeneity following immunological challenges, rather than the expected discrete Th subsets (Azizi et al., 2018; Gaublomme et al., 2015; Kiner et al., 2021). Reminiscent of the paradigm shift in hematopoietic origin (reviewed in section 1), these studies confirm complex populations of $CD4^+$ T cells forming a phenotypic continuum rather than distinct cell states. Regulatory molecules such as cytokines are expressed in overlapping gradients of Teff cell heterogeneity (Kiner et al., 2021) and this heterogeneity is partially shaped by TCR clonotype composition as revealed by multi omics analyses of scTCRseq and scRNA-seq (Azizi et al., 2018; Zemmour et al., 2018). Taken together, scRNA-seq studies have elucidated novel $CD4^+$ and $CD8^+$ T cell subsets with specific cellular functions while proposing alternative pathways for T cell differentiation.

B cells

B cells play a critical role in mediating the adaptive immune response as upon activation, they differentiate into antibody producing plasma cells, which inactivates pathogens, recruits the complement system and triggers opsonization. Furthermore, memory B cells (MBCs) are relatively quiescent cells capable of reactivation upon antigen re-exposure, which triggers differentiation into germinal center B cells or plasma cells (Cyster & Allen, 2019). Immature B cells migrate from the BM to secondary lymphoid organs, where they undergo maturation programs into B1 naïve B cells, or B2 cells including follicular B cells (FOBs) and marginal zone B cells (MZBs) in germinal centers (GCs), all of which provide unique contributions to the antibody response (Cyster & Allen, 2019). scRNA-seq studies, often in combination with single cell B cell receptor sequencing (scBCRseq), have elucidated the underlying mechanisms shaping B cell activation, differentiation, maturation, and cell-cell communications. Recently, the transcriptional switch for intermediate B cells to become FOBs, was identified as metabolic quiescence induced by the activation of the extracellular adenosine salvage pathway (Farmer et al., 2019). Moreover, Farmer and colleagues reported the expression of a low IgM signature in intermediate B cells committing to a FOB fate, which was supported by another scRNA-seq study suggesting IgM expression and signaling determines B cell fate decisions (Tull et al., 2021). The developmental trajectory of intermediate B cells to MZBs was characterized by a high IgM expression, further confirming the regulatory role of IgM in B cell fate commitment (Tull et al., 2021).

In addition, GC B cells, formerly divided in dark zone (DZ) and light zone (LZ) cells, were demonstrated to be more heterogenous than previously thought. scRNA-seq studies identified DZ/LZ intermediates, a LZ GC B cell population transcriptionally capable of proliferation (Holmes et al., 2020), and a novel transcriptionally distinct DZ population termed gray zone (GZ), suggesting a three-cell population model for GC population distribution (Kennedy et al., 2020). It has been suggested that the transcriptional states of GC B cell populations span a continuum rather that separated LZ and DZ states, as a large proportion of GC B cells are transcriptionally intermediate (Holmes et al., 2020; King et al., 2021). Furthermore, class switch recombination (CSR) has been observed in pre-GC B cells (King et al., 2021), supporting the notion that CSR is an infrequent occurrence in mature GC B cells (Roco et al., 2019). Not only BCR affinity, but also CSR influences the antibody-based selection in the GC, as B cell survival is varied between switched and unswitched GC B cells (King et al., 2021). Depending on the antibody class, MBCs displayed differential expression for immune regulatory genes, indicating the activation of unswitched MBCs is highly regulated on a transcriptional level (King et al., 2021). Taken together, scRNA-seq studies have uncovered regulatory mechanisms defining B cell maturation, providing a framework for studying B cell dynamics in health and disease.

Natural Killer cells

NK cells comprise a unique subset of ILCs harboring intrinsic abilities to identify and eliminate target cells independent of human leukocyte antigen (HLA) recognition. Cumulative signals of NK activating and inhibitory receptors determine their killing capacity. The NK cell population represents a dichotomous distribution based on differential expression of CD56 and CD16. The immunomodulatory, cytokine producing $CD56^{bright}CD16^{dim}$ NK population dominates the tissue and differentiates to the more cytotoxic $CD56^{dim}CD16^{bright}$ conventional NK cell population in the peripheral blood (Laskowski et al., 2022). Recent scRNA-seq studies have provided novel insights in the developmental trajectory and function of NK cell subsets. Melsen and colleagues identified a novel $CD56^{dim}$ NK population in the BM characterized by granzyme K (GzmK) expression, which was intermediate of $CD56^{bright}CD16^{dim}$ and $CD56^{dim}CD16^{bright}$ NK cells (Melsen et al., 2022). Whether $CD56^{dim}GzmK^{+}$ NK cells are continuously differentiating towards cytotoxic $CD56^{dim}CD16^{bright}$ NK cells or represent a separate differentiation stage for NK cells has remained elusive, although the authors propose the latter.

Another scRNA-seq study included ~8000 individual NK cells, allowing the identification of several novel NK cell subsets, previously undetected due to cell scarcity in other studies (Smith et al., 2020). Among these novel NK cell populations, one displayed high ISGs and negative CD56 expression and may be specialized for signaling to pDCs and T cells. Furthermore, a rare terminally differentiated NK cell type with loss of ribosomal expression was revealed, suggesting a metabolic switch caused by ribosomal degradation to support cellular activation. In addition, Smith and colleagues identified a $CD56^{dim}$ and $CD56^{bright}$ hybrid NK cell population displaying transcriptional similarity to previously described cytokine-induced memory-like (CIML) NK cells (Romee et al., 2016). This small population of CIML NK cells has not been depicted before as continuously present in human peripheral blood, likely due to the

high abundance of NK cells used in this particular scRNA-seq study (Smith et al., 2020). Lastly, Smith and colleagues reported a higher frequency of adaptive NK cells upon CMV infection, which displayed an altered transcriptional profile specific for improved effector functions upon reinfection. These adaptive NK cells, accompanied with the identification of CIML NK cells, suggest memory-like functions for NK cells (Smith et al., 2020). This was supported by a scRNA-seq study reporting depletion of cytotoxic NK cells in active tuberculosis infection, while adaptive NK cells persisted during latent tuberculosis infection (Cai et al., 2020). Thus, scRNA-seq has uncovered several novel NK cell subsets, proposing alternative cellular functions including a NK cell memory.

Dendritic cells

DCs play a critical role in connecting the innate and adaptive immune system, by processing antigens and presenting them to naïve T cells. DC subtype analysis has remained challenging due to substantial heterogeneity and cell scarcity, which is partially resolved by enabling single cell transcriptomics. A pioneering study by Villani and colleagues introduced a novel perspective in DC research, where six cell types comprise the DC population distribution (Villani et al., 2017). Novel DC subtypes included a proinflammatory subtype within the DC2 population, a rare and poorly characterized DC population with a shared monocyte signature lacking classical DC markers, and most interestingly, a DC subset termed AS DCs capable of a more potent T cell activation than pDCs. The transcriptional profile of AS DCs displayed overlap with DC2 and pDCs, suggesting a relationship between the two cell types (Villani et al., 2017). The characterization of AS DCs has remained controversial as several groups report heterogenous transcriptional profiles representing overlapping DC2 and pDC clusters, reminiscent of AS DC (Alcántara-Hernández et al., 2017; Lukowski et al., 2021; Zhang et al., 2017). Future scRNA-seq studies focusing on the developmental trajectory of AS DCs could shed light on the origin and functional characteristics of AS DCs. The rare monocyte-like DC population identified by Villani and colleagues was later proposed to be a subset of DC2 by single cell transcriptomics and proteomics (Dutertre et al., 2019). This substantial heterogeneity of DC2s was not only observed in the population, but also between individuals as DC2 subset frequency displayed high interindividual variation (Alcántara-Hernández et al., 2017).

Expression of the Autoimmune regulator (AIRE) outside of the thymus has been described before, but the cells expressing AIRE have only recently been confirmed as mature DCs (Fergusson et al., 2019). The differentiation to a mature DC phenotype was accompanied with transient AIRE expression in the absence of tissue restricted antigens (TRAs). This suggests a regulatory role for AIRE expression during DC differentiation and maturation. To summarize, scRNA-seq studies confirm a substantial transcriptional heterogeneity in DC sub type compartments but characterizing functionally distinct cellular sub types has remained challenging.

Challenges and Limitations in the Interpretation of scRNA-seq Data

scRNA-seq technologies allow unbiased and high-throughput sequencing of single cells and have greatly improved our understanding of immune cell heterogeneity in development, health, and disease. Furthermore, the accumulation of scRNA-seq studies over the recent years has allowed for The Human Cell Atlas, a comprehensive cellular reference map with a multiomics level of detail, which allows the systematic study of physiology, pathology, intracellular regulation and cellular communication (Regev et al., 2017). Nevertheless, the computational analyses of scRNA-seq harbors challenges and limitations.

Statistics

The transcriptional estimates are noisier than bulk RNA seq due to material scarcity causing distorted measurements. Zero values, or ‘Dropouts’ are common in scRNA-seq data and indicate only a small fraction of the transcriptome is captured in the analysis at times, leading to failure to detect transcripts due to low sequencing depth or library preparation rather than absent expression reflecting a ‘true zero’ value (Kiselev et al., 2019). These drop out failures have been reduced by droplet-based scRNA-seq experiments, such as Drop-seq, Indrop or 10X Genomics, and introducing unique molecular identifiers (UMIs) (Hérault et al., 2022; Svensson, 2020). Nevertheless, scRNA-seq detects only ~10% of the transcriptome on average, providing a scarce cell transcriptome representation, and sequencing errors in lowly expressed genes may lead to detection variations.

Furthermore, the statistical models reading scRNA-seq data are built under the assumption that the expression level represents a random sampling of a normal distribution, although there have been gene-specific discrepancies observed between the 10x Chromium and Drop-seq platforms (Kharchenko, 2021). However, if the

same protocol was applied to all data, the downstream analysis will not be severely affected by these platform-induced biases (Kharchenko, 2021). Thus, scRNA-seq studies should be aware of the technical flaws and limitations that are inherent to the experimental pipeline.

Dimensions

Differential expression analyses are followed by quantification of cell distance, which represents a measure for transcriptional difference between one cell to another. The high dimensionality of scRNA-seq datasets, accompanied with technical noise, presents a fundamental problem in interpreting scRNA-seq data. Traditional distance metrics (for example, Euclidean, L1, and Canberra), are not able to distinguish distances in a high dimensional data space as they suffer from the 'curse of dimensionality', which describes cell distances becoming more similar as dimensionality increases (Kiselev et al., 2019). This is partially resolved by dimensionality reduction methods that produce a low-dimensional distribution of high-dimensional data, most frequently performed using t-Distributed stochastic neighbor embedding (t-SNE) or uniform manifold approximation and projection (UMAP).

t-SNE converts the high dimensional Euclidean distances to conditional probabilities that represent similarities and uses these probabilities to visualize a neighbor graph representation (Xiang et al., 2021). UMAP builds a weighted k-neighbor graph and computes a low-dimensional representation similar to t-SNE. Both t-SNE and UMAP, but the latter to a lesser extent, focus on preserving local neighboring relationships, thereby often dismissing global distances and distant magnitudes (Kobak & Berens, 2019). This leads to potential loss of biological hierarchy, even though t-SNE and UMAP produce clusters that capture subpopulations and continuous trajectories. Taken together, dimensionality reduction methods play a crucial role in downstream analysis of scRNA-seq data, but researchers should consider the consequences of applying such methods on the interpretation of scRNA-seq data.

Cell type annotation

A key step in scRNA-seq data interpretation is defining cell types from transcriptional clusters. Although cluster groups contain cells with relatively similar gene expression signatures, it is not a given that each cluster corresponds to one or more cell types (Kiselev et al., 2019). Since cells exist in a diverse and plastic transcriptional landscape, a consistent definition of a 'cell type' has remained elusive. Furthermore, transcriptional differences that separate cells into clusters may not possess any biological relevance to separate them into cell types and defining a novel cell type necessitates research beyond transcriptional characterization (Pasquini et al., 2021). Different clustering methods and granularity may yield distinct cluster compositions in the same dataset, leading to a different interpretation of biological cell types.

Under-clustering masks underlying biological structure, thereby losing sufficient resolution to identify distinct biological differences and rare cell types (Pasquini et al., 2021). Contrastingly, over-clustering introduces subsets that are not relevantly biologically distinct, which can be partially resolved by remerging clusters (Patterson-Cross et al., 2021). In the case of severe over-clustering, however, some clusters are 'shattered' and it is therefore no longer possible to reassign clusters following iterative remerging to recover appropriate cell types (Patterson-Cross et al., 2021). Improved ability to distinguish rare cell types often impedes the performance of clustering more frequent cell types (Kiselev et al., 2019), which can potentially be circumvented by reclustering large clusters after an initial clustering (Park et al., 2020; Popescu et al., 2019; Suo et al., 2022). A recent study indicated that current approaches in scRNA-seq studies are often bias towards over-clustering and there are insufficient tools applied to detect over-clustering (Grabski et al., 2022). Although relevant biological variation may be present within these clusters, this over-clustering bias yields false discoveries of novel cell types derived from flawed clusters. Moreover, single cell differential expression analysis displays a high frequency of false discoveries due to replicate variation, contributing to the false characterization of novel cell types (Squair et al., 2021). To minimize incorrect annotation of novel immune cell types, future scRNA-seq studies should focus on a standardized and replicable statistical methodology and researchers should consider whether these cell types provide or impede a deeper understanding of cellular function.

Combined single cell multi omics techniques

Single cell omics techniques vary in their transcript coverage, as some protocols including Drop-seq and SNARE-seq do not read the 5'-end, thereby losing information. Combining sc-multiomics approaches reveals novel insights in cell characteristics. Single-cell sequencing assay for transposase-accessible chromatin (scATAC-seq) reveals the accessibility of chromatin to TFs or other regulatory factors, which generally

represent transcriptionally active genes. The combined analysis of scATAC-seq and scRNA-seq connects the chromatin accessibility at a specific region to the corresponding transcripts, providing temporal cellular information as accessible genomic regions precede active transcription (Dimitriu et al., 2022). Furthermore, comparing scATAC-seq and scRNA-seq data revealed multiple transcriptional states in the same chromatin accessibility profile, thereby uncovering additional regulatory mechanisms (Giles et al., 2022).

scTCRseq can be applied to determine the unique combination of VDJ genes and CDR3 sequence of T cells, which allows studying TCR recombination kinetics, as well as cellular phenotypes and their association with the paired TCR sequence when combined with scRNA-seq (Park et al., 2020; Suo et al., 2022; Tan et al., 2021). scRNA-seq also allows for uncovering potential receptor:ligand interactions between cell types when combined with cell-cell communication (CCC) inference tools such as CellPhoneDB (Dimitrov et al., 2022; Park et al., 2020; Popescu et al., 2019). Spatial RNA sequencing (spRNASeq) combines RNAseq and in situ hybridization, providing additional spatial information with transcriptome data. Cell2location integrates single-cell and spatial transcriptomics, producing comprehensive cellular maps of tissue architectures (Kleshchevnikov et al., 2022; Suo et al., 2022). Thus, scRNA-seq combined with other omics methods can provide additional information that allows for improved characterization of cell types.

Concluding remarks

In this review, we summarized single cell transcriptomic studies that redefined the characterization of immunological cell types over the last decade. Research into immune cell heterogeneity is vital to our understanding of the complex immune cell network in health and disease. The paradigm shift in hematopoietic origin is a prime example of how scRNA-seq can provide a novel perspective on traditional biological views. Furthermore, comprehensive transcriptional maps facilitate research on alternative functions of immune cells, in addition to the classically described function in the prenatal and adult immune system. However, the interpretation of scRNA-seq data includes challenges inherent to the experimental pipeline and researchers should therefore be aware of the biological relevance and meaning of newly characterized immune cell subsets. Researching RNA is inadequate to propose novel functional alterations due to post-translational modifications and limitations in scRNA-seq techniques that impede obtaining a functional characterization. In order to establish a not only transcriptionally, but biologically and functionally distinct cell type, *in vitro* experiments should be performed to support computational claims, or a combination of omics techniques could shape a more complete hypothesis. We expect scRNA-seq to play a central role in immune cell characterization in the upcoming years and it would be exciting to see the complex immunological networks we can uncover by improving protocols and downstream analyses.

List of abbreviations

AGM	Aorto-gonad-mesonephros
BM	Bone marrow
CCC	Cell-cell communication
CS	Carnegie stage
CSR	Class switch recombination
cTEC	Cortical TEC
DC	Dendritic cell
DN	Double negative
DP	Double positive
dpc	Days postcoitum
ETP	Early T progenitor
FACS	Fluorescence activated cell sorting
FL	Fetal liver
GMP	Granulocyte-macrophage progenitor
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
ILC	Innate lymphoid cell
ILCP	Innate lymphoid cell progenitor

iNKT cells	Invariant natural killer T cells
ISG	Interferon-stimulated genes
KLR	Killer cell lectin like receptor
LMPP	Lymphoid-primed multi-potential progenitor
LTi	Lymphoid tissue-inducer
MAIT cells	Mucosal-associated invariant T cells
MBC	Memory B cell
MEM	Megakaryocyte–erythroid–mast cell
MLP	Multilineage progenitor
MPP	Multi potent progenitor
mTEC	Medullary TEC
NK cells	Natural killler cells
NKT cells	Natural killer T cells
NLT	Non lymphoid tissue
pcw	Post conception weeks
pDC	Plasmacytoid dendritic cell
pMHC	Peptide-MHC
RT-Treg	Resident thymic T regulatory cell
scATACseq	Single-cell sequencing assay for transposase-accessible chromatin
scBCRseq	Single cell B cell receptor sequencing
scRNA-seq	Single-cell RNA sequencing
scTCRseq	Single cell T cell receptor sequencing
SP	Single positive
TCR	T cell receptor
TEC	Thymic epithelial cell
Teff	Effector T cells
Texh cells	Exhausted T cell
TF	Transcription factor
Th cells	Helper T cell
Tmem cells	Memory T cell
TRA	Tissue restricted antigen
TRM	Tissue resident macrophage
t-SNE	t-Distributed stochastic neighbor embedding
TSP	Thymus seeding progenitor
UMAP	Uniform manifold approximation and projection
UMI	Unique molecular identifier
YS	Yolk sac

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