



Mind the Map: Technology Shapes the Myeloid Cell Space

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The myeloid cell system shows very high plasticity, which is crucial to quickly adapt to changes during an immune response. From the beginning, this high plasticity has made cell type classification within the myeloid cell system difficult. Not surprising, naming schemes have been frequently changed. Recent advancements in multidimensional technologies, including mass cytometry and single-cell RNA sequencing, are challenging our current understanding of cell types, cell subsets, and functional states of cells. Despite the power of these technologies to create new reference maps for the myeloid cell system, it is essential to put these new results into context with previous knowledge that was established over decades. Here we report on earlier attempts of cell type classification in the myeloid cell system, discuss current approaches and their pros and cons, and propose future strategies for cell type classification within the myeloid cell system that can be easily extended to other cell types.

Keywords: monocytes, dendritic cells, human peripheral blood, multidimensional, single-cell RNA sequencing, mass cytometry

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INTRODUCTION

Cell-type identification is an integral part of current immunology (1–5). The immune system as an organ is an assembly of an incredibly complex network of different types of immune cells including T and B lymphocytes, NK cells, innate lymphoid cells, monocytes, macrophages, and dendritic cells (DC), granulocytes including neutrophils, basophils and eosinophils, and mast cells (6). These cell types have specialized roles during homeostasis and infection. Moreover, it became clear that each of these significant immune cell types consists of cell type-specific cell subsets, for example, three monocyte subsets have been described in human peripheral blood, the so-called classical, intermediate, and non-classical monocyte (7). To understand the individual role of each of these subsets, it is crucial to understand the full heterogeneity of these cell types and their subsets to pinpoint the dedicated functions (8). This also needs to be considered in a spatiotemporal fashion, since immune cells are influenced in their function by their respective microenvironment as well as over time (9–11). For example, monocytes accumulate in peripheral reservoirs under homeostatic conditions, but during inflammation, they exert primarily pro-inflammatory effector functions (11–13). At a later time point during the repair phase of an inflammatory response, monocytes are characterized by regulatory properties necessary for tissue repair (14). During the last decade, technological advancements have been used to further refine our understanding of the diversity of cell types and subgroups within the immune system (15). These novel technologies must be put into context with the traditional way of defining cell types mostly relying on low-dimensional data

including microscopy, functional assays, and expression of single marker genes. In the first part of the review, we discuss the current principles and strategies of defining cell types and subsets, while highlighting the different aspects of resolving cellular heterogeneity. Here we want to outline how these principles have been applied to the DC/monocyte cell space. Moreover, we will provide a framework for the integration of these recent technological advances to define cell types, subsets, but also functional states of these subsets in an iterative process.

THE MONONUCLEAR MYELOID CELL SPACE AS AN EXAMPLE FOR CELL TYPE DEFINITION

Monocytes and DC arise from the myeloid lineage of the hematopoietic system and make up about 11% of human blood leukocytes (monocytes ~10%, DC ~1%). In humans, monocytes and DC are defined as MHCII⁺CSF-1R⁺ cells, mostly generated through a cascade of continuously differentiating progenitors in the bone marrow. The last shared intermediate is the monocyte-DC progenitor, MDP, which is characterized as a CD45RA⁺CD123^{int}CD115⁺ fraction of a heterogeneous granulocyte-monocyte progenitor (GMP) population (16). Using CLEC12A and CD64 expression, a focused monocyte progenitor without DC potential, the common monocyte progenitor (cMoP), was described recently (17). This restricted precursor differentiates via pre-monocytes to monocytes, which in mice egress the bone marrow in a CCR2-dependent fashion (18).

Monopoiesis is highly dependent on the hematopoietic growth factor receptor CSF-1R and is enhanced, especially during infection or “sterile” inflammation (19–22). This phenomenon highlights the function of blood monocytes, which mainly serve as a reservoir for tissue-residing monocyte-derived macrophages and monocyte-derived DCs, especially during inflammation. Under homeostatic conditions, the majority of monocytes are weak phagocytic cells and are less efficient in antigen presentation when compared to DCs and macrophages (14, 23).

Initially described by Steinman and Cohn in the early 1970s DC have been extensively studied in recent decades (24, 25). Nevertheless, the high variability regarding ontogeny, phenotype, tissue localization, and function has hampered to find a comprehensive description of this cell type for a long time. On a functional level, DC are very efficient in phagocytosis and antigen presentation and are therefore crucial for the initiation of an adaptive immune response (23). DC are generated from MDPs giving rise to DC-committed precursor cells called common DC progenitors (CDP) which serve as precursor for plasmacytoid DCs and the two classical DC subtypes cDC1 and cDC2 (26, 27). Recently, a cDC-restricted progenitor cell, the pre-cDC, has been described in mouse and human (5, 28–30). Concerning pDCs, a new model has been recently suggested (1, 31). In fact, 70–90% of pDCs seem to be IRF8-dependent and derive from a different pre-pDC precursor. These cells actively produce type I interferons and do not present antigen very well. Further studies are required to corroborate these recent findings.

WHICH ASPECTS DEFINE CELLULAR IDENTITY?

The Traditional Approach: Morphology, Phenotype, and Function

Several characteristics have been used to describe and define cell types and subsets. Initially, morphological characterization by early microscopy and functional observations laid the ground for the idea of different categories of cells. Primarily, features like size, shape of the cell, and/or nucleus, density, and staining behavior for specific dyes were used to separate immune cells into several cell types and subsets (24, 32–37).

Collectively described as mononuclear phagocytic cells, macrophages and monocytes were defined by their unique morphology and ability to take up pathogens and debris (32, 33, 38, 39). Several experiments suggested that blood-derived monocytes will give rise to different types of tissue-resident macrophages, which was comprehended by van Furth and Cohn as the “mononuclear phagocyte system” (MPS) (40). Later, Ralph Steinmann described cells that display a characteristic morphology when cultured on glass surfaces (24). Due to their morphology, he termed them dendritic cells. These DCs were quickly found to be professional antigen presenting phagocytes and were incorporated into the definition of the MPS (25, 41, 42).

The MPS has been defined based on morphology and shared functionality of monocytes, DCs, and macrophages as a broader framework to describe the role of these cell types during homeostasis and immunity. However, the original definition of the MPS cannot adequately explain the heterogeneity of these cell types concerning their origin, tissue localization, disease association, regulation, and function. For example, contrary to the original ideas, blood monocytes are not the only reservoir for tissue-resident macrophages. An enormous body of research established that tissue-resident macrophages are mostly generated by early progenitors during embryogenesis and exhibit to a limited extent the partial ability for self-renewal (43–47). Only some tissues of barrier organs like the intestine rely on the replenishment of tissue-resident macrophages by differentiation of monocytes during adult life, especially during infection or inflammatory conditions (48). Nevertheless, when looking at monocyte-derived and tissue-resident macrophages, we must acknowledge that these cells have a high phenotypic and functional similarity. This redundancy is essential for the (functional) replacement of yolk-sac derived tissue-resident macrophages in some tissues but makes it difficult to find a unified classification.

The use of surface marker detection by monoclonal antibodies and flow cytometry has revolutionized the way of cell type definition throughout immunology. While a functional heterogeneity of monocytes was suggested by several earlier studies (34–37, 49), it was two-color flow cytometry that provided a tool to clearly define two major monocyte subsets by their expression of CD14 and CD16 (50, 51). About 80 to 90% percent of peripheral blood monocytes express CD14 but lack the expression of the Fcγ-receptor III (FcγRIII/CD16). This subset is characterized by a higher phagocytic activity compared to the minor subset expressing CD16 and intermediate levels

of CD14. Also, CD16⁺ monocytes can be further separated based on their expression of CD14 into CD14^{dim} CD16⁺ population and a less frequent CD14⁺ CD16⁺ subset (52, 53). The CD14⁺CD16⁻ subset of monocytes is referred to as classical monocytes, monocytes expressing CD14 and CD16 as intermediate monocytes and non-classical monocytes are defined as the CD14^{dim}CD16⁺ subset (7, 52, 53). Furthermore, during the last decade, several markers have been suggested for defining the monocyte cell heterogeneity, including Slan and CD2/FcεRI (54–56). However, these markers do not reach the specificity that would be required for an unambiguous definition of cell types or cell subsets (also see below and **Box 1**).

Like monocytes DC have been first described on the basis of their morphological and functional aspects. Here, pDCs are characterized as main type-I interferon (IFN- α/β) secreting cells with plasma cell-like morphology (57). Activation and secretion of type-I interferons are facilitated by recognition of virus-derived nucleic acids, especially by endosomal nucleic acid-sensing Toll-like receptors (TLRs) TLR7 and TLR9 (57). Initially, these cells were identified by several groups under different names, including natural interferon-producing cells, plasmacytoid monocytes, and plasmacytoid T-cells (58–61).

Finally, a consensus name, the plasmacytoid DC was introduced and phenotypic markers were defined including human blood dendritic cell antigen (BDCA)-2, human IL-3R α (CD123) and BDCA-4 (57, 62–65). However, as already mentioned before and described in more detail later, previously reported experiments suggest that this consensus is once again challenged (1, 31) strongly arguing for an iterative process of cell type definition continuously including new information.

Besides pDCs, there are two subsets of myeloid or classical DC (mDC/cDC) that can be distinguished in the Lin⁻MHC-II⁺CD11c⁺ fraction (66, 67) by using the non-overlapping markers CD1c (BDCA1) or CD141 (BDCA3) in flow cytometry (64, 65). These DC subsets have been termed cDC1 (CD141⁺ DC) and cDC2 (CD1c⁺ DCs), respectively, which have been reviewed extensively elsewhere (68–73). While these classical markers are widely used, further markers have also been suggested for subset classification of DCs (73, 74). For instance, CD141⁺ cDC1 can be identified by using antibodies against XCR1 (75, 76), CLEC9A (77–79) and CADM1 (80). Interestingly, all DC populations vary regarding their expression of the pattern recognition receptor family toll-like receptors, which is highly correlated with the functional roles these cells play in T-cell

BOX 1 | Proposed framework for the definition of cell types, cell subsets, and functional states of cell types and subsets.

Cell type definition based on a single parameter space (e.g. only ontogeny) will be inferior to integrated approaches utilizing additional information (ontogeny, -omics data, phenotypic, and functional data). Nevertheless, even with such a large heterogeneous parameter space at hand, cell type definition is still not trivial. We propose a framework to define cell types and their subsets that is based on knowledge from decades of developmental and cell biology, further substantiated with recent developments and results in the field of single-cell omics (165–168). Certainly, such proposal will require larger community involvement and is mentioned as a starting point for discussion. This principle can be extended to define other cell types as well.

According to this framework, “**cell types**” would be defined as follows:

“Cell types” constitute the highest category. Cell types are defined by the lack of transdifferentiation capacity in more than 95% of all physiological and non-physiological conditions. Furthermore, cell types exhibit certain phenotypic, functional and genome-wide (transcriptome, epigenome, other) characteristics that are unique to all cells of a particular type. For immune cells that are terminally differentiated, cell types would include T and B lymphocytes, NK cells, monocytes, macrophages and DC, neutrophils, basophils and eosinophils, mast cells and innate lymphoid cells. For the stem cell and precursor compartment, the hematopoietic stem cell would be one cell type, while all precursors could be another cell type. Particularly in the precursor space, more research is required to define whether—based on this definition—further cell types or only cell subsets (see below) exist. This is similarly true for cell type development during embryogenesis. However, such a framework would certainly guide future research, specifically exploiting experimental systems that would allow answering the question, whether a cell is still capable of transdifferentiating toward another cell type.

“**Cell subsets**” would be defined as follows:

“Cell subsets” are a secondary category within any given cell type. Cell subsets share certain phenotypic, genome-wide (transcriptome, epigenome) and functional features within a given cell type, but are distinct in other phenotypic, functional, or genome-wide features that are unique to them within a cell type. In an ideal setting, these features should not overlap with those features that characterize the cell type. Furthermore, the feature set characterizing a cell subset should not change if cells are analyzed from different compartments (tissues, organs) and under differing conditions (homeostasis, acute inflammation, repair phase, etc.). Cell subsets can be further distinguished from cell types in that cell subsets can change into another subset of the same cell type to the degree that is higher than 5%. For example, it is known that classical monocytes can further differentiate into non-classical monocytes via the intermediate monocyte subset.

“**Functional states**” are defined as follows:

“Functional states” are the overall current program of any given cell. Again, “functional states” would be defined by a specific pattern of phenotypic, functional and genome-wide characteristics, which ideally would exclude features characterizing cell types or subsets. “Functional states” rely on spatiotemporal information (e.g., location, the cell’s individual age, the age of the organism), the activation state (homeostasis, acute, chronic inflammation, repair phase, etc.) and any combination thereof. Clearly, “functional states” can only be defined by integrated approaches and patterns or signatures of many parameters. Single parameter definitions for functional states are very unlikely. Any given cell can be described by combinations of “functional states.” In other words, “functional states” can be linked to intracellular biological modules responsible for different cellular functions. A cell could express pro-inflammatory cytokines and have elevated migratory capacity. “Functional states” can even be shared among different cell types and cell subsets. However, together with the definition of the cell type and subset, a cell can be defined unambiguously according to the three levels of cell type classification.

“Cell types,” “cell subsets,” and “functional states” will be governed by transcriptional programs that are linked to defined and specific networks of transcription factors (TFs) not only single TFs. Therefore, the description of such networks might be another means of defining cells accordingly.

The introduction of functional states will reduce the excessive introduction of new cell types or subsets and—in our view—also represents the well-known plasticity of the myeloid cell space better.

activation. For example, human CD141⁺cDC1 cells express high amounts of TLR3 (81), a pattern recognition receptor highly associated with cross-presentation (82) and thus cDC1s are specialized in presenting intracellular antigens to CD8⁺ T-cells in human and mice (83).

The most abundant subset of blood DCs are CD1c⁺ cDC2s, which can be defined analytically by expression of CD11c, CD1c (BDCA1), and FCεRIa (54, 64, 84). Furthermore, CD1c⁺ cDC2 express high levels of class II MHC molecules like HLA-DR, HLA-DQ, and show a high endocytic capacity, which specializes this DC type for the presentation of exogenous antigen to CD4⁺ T cells (64, 84). As we will outline below, future work will require community efforts to integrate the differential usage of cell subset classification markers to generate consensus nomenclatures.

Collectively, the definition of cell types of the MPS and their subsets was initially based on cellular morphology, further developed by introducing immunophenotyping using antibodies against the respective cell surface markers and complemented by a functional assessment of the cell subsets identified. We spare the many controversial findings throughout this period, which only reflects the limitations of these approaches to generate a widely accepted nomenclature of cell types and subsets.

Ontogeny as a Concept for Cell Type Definition

A group of leading experts in the field of monocyte, DC, and macrophage biology has recently proposed a nomenclature, which is based mainly on the ontogeny and tissue localization of cells (73). The proposed two-level model defines a cell type, first by its origin (level 1), which is further improved by adding a functional, phenotypic or location information (level 2) of the particular cell type. This aspect of cell type classification and the ontogeny of DCs and monocytes have been reviewed extensively (48, 72, 85).

The usage of cellular origin for cell type classification is beneficial since such approach already segregates distinct, functional units. For example, it was suggested that all phagocytes that are generated by yolk-sac derived progenitors should be referred to as macrophages and cells derived from the hematopoietic lineage as monocyte-derived cells (8, 68). A further advantage of using origin and development of immune cells as a guiding principle for cell type definition is the conservation of ontogeny across species. However, although there is a substantial overlap of ontogenies in human and murine macrophage, monocyte and DC development, there is also considerable disagreement (16, 83, 86–88). Additionally, the ontogeny of myeloid cells is difficult to study in humans, and most results are obtained by mice experiments and then projected to human myeloid cells. Clearly, the ontogeny approach is a very important aspect of cell type definition, but it needs to be combined with other characteristics of cells.

HIGH-DIMENSIONAL APPROACHES SHAPE THE MYELOID CELL SPACE

Here, we introduce the latest technological advancements that have made substantial contributions to clarify the monocyte/DC

compartment. Furthermore, we want to discuss open questions and challenges associated with these new technologies. Multi-dimensional approaches have significantly improved our understanding of the myeloid cell space by providing more features resulting in higher resolution for cell typing. To contextualize this, we want to provide examples that outline how high-dimensional methods have shaped our understanding of heterogeneity in human blood-derived monocytes and DC.

Although conventional flow cytometry has revolutionized cell type classification, it is limited in the number of parameters (markers <20) being analyzed at the same time. In the early 2000s, there were a couple of technological advancements that paved the way to the development of mass cytometry enabling parallel analysis of up to 40 parameters (89–93). This higher depth of data simultaneously enabled a multitude of possibilities for immunological and biomedical sciences, including the high-dimensional assessment of cross-patient cell type dynamics during acute myeloid leukemia (94–97). More recently, multi-color flow cytometry (MCFC) has been introduced, increasing the parameter space to a similar range, as seen in mass cytometry. However, although mass cytometry and MCFC allow high-throughput protein profiling of thousands of cells, the restriction to <40 protein markers may be underrepresenting the true number of variables that are necessary to define the heterogeneity in highly complex biological samples. Besides, these markers have to be selected *a priori*, which may put a bias on the results obtained by mass spectrometry or MCFC. Another revolution was introduced by the development of high-throughput gene expression profiling methods like microarray-based technologies and RNA-sequencing enabling to profile thousands of genes in a single sample (98, 99). This second genomic revolution enables the genome-wide assessment of gene expression, which not only allows to characterize cellular subsets but also to investigate regulatory networks (20, 100–102).

One of the first studies that performed microarray analysis of human DCs compared the transcriptomes of sorted cDC1, cDC2, and pDCs populations from peripheral blood and tonsils to deeply characterize these subsets (103). Robbins et al. performed a comparative study to put the transcriptome data of DC subsets into context of other myeloid and lymphocyte populations in blood (104), which resulted in the identification of important conserved signature genes, thereby strengthening cDC1, cDC2, and pDC as distinct DC subsets. Moreover, assessing transcriptomic data of both murine and human immune cells allowed to align DC subsets across species (104, 105). Another important study performed transcriptome profiling of human blood CD14 and CD16 monocyte populations, three DC subsets pDC, cDC1, and cDC2 as well as their skin counterparts cDC1, cDC2, and skin derived CD14⁺ cells (80).

Notably, cell types like skin cDC1 and cDC2 grouped together with their counterparts isolated from blood, suggesting a high similarity of DC subsets independent from the microenvironment. We extended these findings to compare different DC subsets in many individuals and different tissues [lymphohematopoietic (blood, thymus, spleen) and non-lymphohematopoietic (skin, lung)] allowing to characterize the impact of the microenvironment on the identity of a cell

type (74). Integration of immune phenotyping, gene expression profiling, and bioinformatic analysis revealed that DC subsets from blood, spleen, and thymus were transcriptionally conserved, with only minor transcriptomic differences between the same DC subsets across tissues. In contrast, the transcriptomic consequence of the respective microenvironment was stronger in lung and skin subsets. This suggests a higher tissue imprinting of non-lymphohematopoietic DC subsets in barrier organs like lung and skin, when comparing to the tissue imprinting that has been reported for tissue-resident macrophage subsets (47, 100, 106, 107). However, the difference between different DC subsets (cDC1 vs. cDC2) is still larger than the differences between the same DC subset among different tissues (e.g., skin cDC1 vs. blood cDC1).

Collectively, gene expression profiling and comparative bioinformatic analysis have substantially contributed to understand the complex DC networks across species further improving current descriptions of unified and more unbiased classifications (73, 105, 108).

Early transcriptomic approaches of human and mouse monocyte subsets not only helped to deeply characterize these cell types but also presented a framework to validate high conservation of gene expression profiles between mouse and humans (104, 109). For example, a combination of well-designed functional assays and gene expression profiling helped to refine the role of non-classical monocytes as the counterpart to murine “patrolling” Gr1⁻ monocytes (110). Other studies sharpened the definition of the intermediate and non-classical monocytes as distinct cell subset (110–112). Interestingly, these studies revealed a high similarity of non-classical and intermediate monocytes, underlining the transitional nature of these cells, as they show intermediate expression for most of the marker genes differentially expressed between classical and non-classical monocytes. Interestingly, a unique module of class-II MHC genes was highest expressed in the intermediate monocyte population (111).

Measuring RNA rather than protein levels represents one of the major limitations of gene expression profiling methods. While the overall correlation of transcriptome and proteome is relatively high (113, 114), RNA-seq and microarrays do not allow to assess post-translational modifications, which represent a central part of cellular regulation (115, 116). To overcome this limitation, mass cytometry has been utilized to profile post-translational modifications like phosphorylation, methylation, and glycosylation (117, 118). A good example of the value of methods with larger feature size compared to single or few marker studies is the definition of cells expressing the carbohydrate modification 6-Sulfo LacNAc (*Slan*) on the PSGL1 protein. Indeed, myeloid cells presenting *Slan* initially were termed “*SlanDCs*” (119–121), while others described an overlap of *Slan*⁺ cells with non-classical monocytes (122, 123). However, all these studies largely rely on low-dimensional marker assessment by flow cytometry and are not always directly comparable due to differences in their choice of markers or gating strategies. To investigate this in a more unbiased fashion, Roussel et al. defined a 38-marker panel to study human myeloid cells from peripheral blood by mass cytometry

(124). A semi-supervised analysis of the data resulted in the identification of distinct monocyte populations, two subsets overlap with markers from classical and intermediate monocytes while there are two subsets of monocytes that are similar to non-classical monocytes. The multi-dimensional analysis maps *Slan*⁺ cells to the non-classical monocytes and does not show alignment with any DC population. In this study, *Slan* separates the non-classical monocytes into a *Slan*^{high} and a *Slan*^{low} CD14^{dim}CD16⁺ population. However, earlier genomic comparisons of sorted *Slan*^{high} vs. *Slan*^{low} subsets did not reveal a significant difference between those two populations (110). More recently, by combining index sorting and high-content single-cell RNA-sequencing, we show further evidence that *Slan* expression does not reflect different cell subsets as the underlying overall transcriptional program is not different between *Slan*^{high} and *Slan*^{low} cells. Moreover, we clearly show that *Slan*⁺ cells are all non-classical monocytes (125).

Manual gating of monocytes by CD14 and CD16 is biased by the investigator, which is a disadvantage for large multi-center clinical studies. Unsupervised and semi-supervised computational analyses improve the accuracy and reproducibility of subset definitions (95, 117, 124, 126–128). However, interpretation of these results must be performed with special care, since the primary analysis is still dependent on manual parameter settings by the investigator. For example, in contrast to an earlier study utilizing mass cytometry (124) similar profiling of human mononuclear myeloid cells revealed three subsets of human monocytes in two other studies, while others report significant heterogeneity including three non-classical, one intermediate and four classical subsets (22). Interestingly, Hamers et al. identified a non-classical population, which is quite different to other non-classical populations and expresses CD9⁺ CD41⁺ and CD61⁺, which may represent an eosinophil/basophil contamination (129–131). Another interesting observation is the rather low inter-individual difference of human monocyte populations during homeostasis when assessed by mass cytometry (22, 132).

High-throughput gene expression profiling by microarray or RNA-seq has paved the way to understand the regulatory networks within human monocytes and DC. These technologies are indispensable for high-depth characterization of immune cell types. Nevertheless, these population-based methods are not designed to detect further cellular heterogeneity within a sample. The gene expression measurement in a population-based RNA-seq represents an average signal of typically more than 10,000 individual cells, resulting in leveling out any further heterogeneity. Frequently, samples are generated by flow cytometry assisted cell sorting, which relies on the information of a limited set of marker genes. However, if these markers are not sufficient for detecting the full heterogeneity of the tissue, the results may be underestimating the true heterogeneity.

Transcriptional profiling of individual cells by single-cell RNA-seq has been introduced in 2009 (133, 134) and has revolutionized cell type discovery in all fields of biology (135–142), therefore it may be claimed as “third genomic revolution.” Single-cell RNA sequencing approaches allow transcriptional profiling of 10,000s of individual cells. In contrast

to population-based RNA-seq, the groups of cells are not defined *a priori*, rather the cell classification is based on the similarity of gene expression profiles.

A series of studies applied single-cell RNA-seq to understand the heterogeneity of human blood DCs and DC progenitors (5, 30, 143). See et al., as well as Villani et al., detected and characterized the conventional subsets, including cDC1, cDC2, and pDC. Surprisingly, beyond these similarities the results differed significantly, strongly arguing that such high-dimensional data require particular care when assigning cell types and cell subsets. We defined cell types and subsets by a combination of function, phenotype and transcriptional profile, which lead to the identification of precursors (pre-cDCs) for the cDC1 and cDC2 subsets in addition to the three main DC subsets (5). To reconcile these two major initial reports, we developed a strategy that allows developing cell type classification consensus based on phenotypic and transcriptional features also including prior knowledge (125). This approach revealed that (1) the AXL⁺Siglec6⁺ DCs (AS-DCs) described by Villani et al. are mainly pre-cDCs as described in (5), (2) Mono4 are contaminating CD56^{dim} NK cells, and (3) cells introduced as CD16⁺ CSF1-R⁺ CTSS⁺ DCs are not belonging to the DC lineage. This general strategy is not restricted to myeloid cells but can be applied to any cell type classification problem in any species (125).

Recently, single-cell RNA-seq has also been used for improving our knowledge about the generation of DCs from bone marrow-derived progenitors. There is evidence that there is much higher flexibility in the development of DC and monocytes than already appreciated. Hematopoietic models that are not based on repeating rounds of division and differentiation (72, 144, 145) allow for incorporation of recent findings that suggest that cDCs can be generated by lymphoid progenitors (146). Also, the latest reports show important evidence that the large majority of pDCs arise from lymphoid progenitors rather than CDPs (1, 31). Probably, a community effort to clarify future naming and nomenclature of these cells is now warranted. Importantly, the recent high-dimensional characterization of pDCs (5, 125, 132, 143) and new insights into their ontogeny in mice (1) could form the basis for such new discussions.

Clearly, this is only the beginning of applying these technologies to open questions concerning the plasticity of the myeloid cell compartment. We also recognize that single-cell RNA-seq data are currently challenging our view on cell type classification and function within the myeloid cell compartment. However, in the long run, we are convinced that the higher information content per cell will give us a much better understanding of individual cells within any given tissue, organ, or inflammatory response.

PROPOSAL OF GENERAL PRINCIPLES FOR CELL TYPE DEFINITIONS

Considering the apparent ease, with which different cell types were characterized based on morphological differences a century ago (39), our capabilities to simultaneously measure hundreds to thousands of parameters per single cell seem to decrease

our ability to agree on defined cell types and cell subsets (1, 5, 31, 143). The ability to detect heterogeneity between individual cells has extended to biological differences that are not related to questions concerning cell type or cell subset. The best-characterized biological process in single cell –omics data being cell cycle in proliferating cells (147–149). Certainly, cell cycle differences should not classify two cells of the same type as different cell types or subsets. Stochastic behaviors of single cells, e.g., in transcription (150, 151) would be another biological phenomenon that should not impact on cell classification aspects. Furthermore, data sparsity, still very apparent in all sequencing-based single cell technologies, requires attention, when dealing with cell type definitions.

Similarly, important is the question, whether all biased approaches requiring feature selection (e.g., which markers to be analyzed) prior to analysis are good starting points for cell type definitions. These would include all multi-color flow cytometry and single-cell mass spectrometry approaches. Potentially a more appropriate approach would be the combination of markers (chosen by the investigator) with unbiased approaches provided by single cell sequencing-based technologies. This is crucial since it allows to link the enormous body of research that has been performed with flow cytometry-defined cell populations (e.g. ontogeny) with results obtained by analysis of high-dimensional data. For example, index sorting based on previously defined cell surface markers combined with scRNA-seq might be a better way of defining the cell population structure as well as the practicality of certain protein markers to capture the population structure (125, 152, 153). Alternative but significantly more expensive approaches are based on the combination of full transcriptome scRNA-seq and oligonucleotide-labeled antibodies (154, 155). It can be expected that these approaches require iterations of experiments until markers are identified that truly reflect the underlying population structure. In this context, it is important to note that even such large endeavors such as the Human Cell Atlas will require the integration of additional layers of information in addition to scRNA-seq data. Furthermore, we postulate that these iterations will lead to consensus maps as a basis for cell type definitions (125). Very much like the cluster of differentiation (CD) workshops for antibodies (156), a community effort will be necessary to agree on the different versions of such consensus maps of individual cell types.

However, even if the combination of truly unbiased single cell –omics approaches and antibody-based techniques leads to novel consensus maps of immune cells including the myeloid cell space, we propose that each cell type and more importantly each cell subset requires to be functionally characterized, as we have previously demonstrated for human DCs in blood (5). In other words, we strongly argue that a final definition of a cell subset should be validated on functional differences and not only on transcriptional and phenotypic differences.

Once cell types are defined under homeostatic conditions, which is a major goal of the Human Cell Atlas (157), an even more daunting task will be to define cell types and subsets under pathophysiological processes. While certain cell types will be under developmental trajectories (cell states) under physiological conditions, the space for different cell states in disease settings will further increase (158). More importantly, under these

conditions, there will be mainly changes in parameters related to biological function rather than features defining cell types or subset. A major goal for further cell type definitions will be to integrate these functional states and trajectories. In this context, we propose cell types as the highest level to distinguish cells. For example, DC, monocytes, and macrophages would qualify as individual cell types, while pDC, cDC1, and cDC2 would qualify as DC subsets (5, 125). Each of these subsets can exist in different functional states that depend on location, differentiation stage, acute or chronic activation signals, to name only a few (69, 74). Again, even for functional states, we would propose to define cells based on hundreds of parameters measured by single cell –omics technologies to be combined with classical marker strategies but finally also integrate functional readouts for these cellular states.

Even if we can agree on such an approach, the question remains, how this can be realized technically? In fact, this is not a mere technical question, as it requires to consider methods that are more independent of investigator bias. For example, we strongly suggest building approaches that will allow us to build cell type definitions based on machine learning rather than on investigator-driven and individualized analysis pipelines. Single-cell transcriptomics algorithms as they are implemented in singleR (159) or scMatch (160) are good starting points. Nevertheless, they still heavily rely on an investigator's interpretation of such high-feature data spaces. Cell type definition could be a classification problem requiring the respective machine learning as they are used for classifier generation in other areas (161, 162). We do not favor solely data-driven machine learning but would suggest the integration of prior knowledge. First attempts to develop such methods are currently underway, and we will soon know, whether the introduction of machine learning based cell class prediction will truly aid our attempts to make sense of the hundreds to thousands of parameters that we now can routinely measure from single cells.

SUMMARY AND OUTLOOK

Since the discovery of myeloid cells more than a century ago, we have learned a lot about these important immune cells. Their

enormous plasticity is fascinating and challenging at the same time. Not surprisingly, cell type definitions and nomenclature—up to the day—have been changed or updated regularly (48, 68, 108, 163, 164). A unified nomenclature is the basis for an effective communication among scientists and will accelerate discovery of novel therapeutics. Moreover, high-dimensional profiling of samples will facilitate to compare results and cell types across experiments, tissues and species. Even with the highest number of parameters known per any given cell, we still differ in our interpretations of certain cell types within the myeloid compartment. While it will be rather critical to include prior knowledge when labeling cells based on high-dimensional single cell data, we need to develop better tools based on robust mathematical rules that help us to determine cellular phenotypes and functions less ambiguously. With the emergence of powerful machine learning and AI-based methodology, the time has probably come to utilize such approaches to our benefit when describing cell types, cell subsets, and their functional states. Irrespective of the power of such approaches, we also need to accept that we are far from a complete understanding of these cells. Additional layers of information, for example, epigenetic information, will have to be included in cell type definitions as they arise. Therefore, we foresee numerous iterations of defining cell types and their functions in the decades to come. In other words, consensus maps of cell types and subsets that we agree on today will form the basis for newer maps with updated information content in the future. A potential framework for such a community-based effort has been outlined here.

AUTHOR CONTRIBUTIONS

PG and JS conceived and wrote the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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