

# Emerging single-cell technologies in immunology

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## ABSTRACT

During evolution, the immune system has diversified to protect the host from the extremely wide array of possible pathogens. Until recently, immune responses were dissected by use of global approaches and bulk tools, averaging responses across samples and potentially missing particular contributions of individual cells. This is a strongly limiting factor, considering that initial immune responses are likely to be triggered by a restricted number of cells at the vanguard of host defenses. The development of novel, single-cell technologies is a major innovation offering great promise for basic and translational immunology with the potential to overcome some of the limitations of traditional research tools, such as polychromatic flow cytometry or microscopy-based methods. At the transcriptional level, much progress has been made in the fields of microfluidics and single-cell RNA sequencing. At the protein level, mass cytometry already allows the analysis of twice as many parameters as flow cytometry. In this review, we explore the basis and outcome of immune-cell diversity, how genetically identical cells become functionally different, and the consequences for the exploration of host-immune defense responses. We will highlight the advantages, trade-offs, and potential pitfalls of emerging, single-cell-based technologies and how they provide unprecedented detail of immune responses. *J. Leukoc. Biol.* 98: 23–32; 2015.

## Introduction

Infectious diseases are the phenotypic expression of microbial encounters with host-immune systems. Therefore, detection of microbes is an essential function of the immune system with the ultimate objective to eradicate or confine the invasive pathogen. The immune system consists of 2 arms: innate, which is the ancestral element of the immune system, and adaptive, which evolved more recently [1, 2]. The main distinction between the

2 arms is that innate cells recognize general patterns of microbes with germline-encoded sensors, whereas adaptive-immune cells undergo gene rearrangement to create unique receptors. The initial steps of the host-defensive response are carried out by innate immunity, which senses microbial intruders via soluble molecules present in the extracellular milieu (primarily acute-phase proteins) of the host external barriers (skin and mucous membranes) that are in close contact with the natural environment. Innate-immune cells are capable of recognizing a great diversity of exogenous (microbial products, inert particles, or compounds) and endogenous (membrane-associated or cytoplasmic molecules, nucleic acids) danger signals. MAMPs or PAMPs comprise numerous bacterial (LPS, lipopeptides, fragments of peptidoglycan, flagellins, unmethylated CpG sequences, lipids, and nucleic acids), viral (5'-triphosphate ssRNA, dsRNA, ssDNA, dsDNA, and circular DNA), fungal (mannans,  $\beta$ -glucan, and chitin), and parasitic (surface sugars, glycosylphosphatidylinositol, *Plasmodium falciparum*-encoded erythrocyte membrane protein 1) molecules. Non-microbial danger signals comprise foreign compounds (silica, alum) and endogenous molecules (RNA, DNA, ATP, and uric acid), collectively known as damage-associated molecular patterns. These diverse molecular patterns are sensed in a quite specific manner by sets of PRRs expressed at the cell surface or intracellularly. Members of the PRR family include TLRs, C-type lectin receptors, nucleotide-binding oligomerization domain-like receptors, the inflammasomes, retinoic acid-inducible gene I-like receptors, and the scavenger receptors [3–6]. Upon binding of their cognate ligands, the PRRs activate several cascades of signal transduction, primarily the NF- $\kappa$ B, the MAPK, and IFN regulatory factor pathways, resulting in the transcription of a multitude of immune genes [7–9].

Recognition of MAMPs/PAMPs by PRRs is also implicated in the binding, engulfment, and killing of microorganisms by myeloid phagocytes (i.e., macrophages, DCs, and neutrophils) [10]. Following phagocytosis, APCs, primarily DCs and macrophages, process MAMPs/PAMPs into fragments and migrate to lymphoid tissues, where they present antigens to cells of the

Abbreviations: DC = dendritic cell, IEC = intestinal epithelial cell, MAMP/PAMP = microbial- or pathogen-associated molecular pattern, PRR = pattern recognition receptor, qRT-PCR = quantitative real-time PCR, RV = rota virus, SPADE = spanning-tree progression analysis of density-normalized events

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adaptive-immune system (T and B cells). Adhesion and costimulatory molecules expressed at the cellular surface and mediators (cytokines, chemokines, IFNs, and other immune activators and regulators) released in the extracellular environment are also key players in the orchestration of adaptive-immune responses by the innate-immune system [11–13]. The separation of the innate- and adaptive-immune system is not always clear cut. Innate lymphocytes, such as NK cells, and adaptive lymphocytes, such as B cells, exhibit innate- and adaptive-immune characteristics. For example, NK cells are considered to be part of the innate branch of the immune system but also have been shown to possess some form of memory, a feature generally attributed to the adaptive immune system [14].

A vast array of proinflammatory and anti-inflammatory immune mediators exerts counter-regulating activities meant to keep the magnitude of the inflammatory response and its timely resolution under control [15]. Qualitative, quantitative, or time-dependent deregulation of immune responses may have dramatic consequences for the host [16, 17]. Inherited functional mutations of immune genes or acquired immunodeficiencies may promote microbial growth, infection, and sepsis. Conversely, genetic mutations may also result in exuberant inflammatory systemic responses, which may induce life-threatening, multiple organ dysfunctions. Therefore, tight regulation of immune responses is critical for the maintenance of homeostasis and for the prevention of infection or autoimmune diseases.

During evolution, microbes have been powerful drivers for the selection of genes associated with resistance to infection and diseases. Genetic diversity has been shaped by constant interactions between microorganisms and their hosts within evolving ecosystems [16, 18]. Therefore, immunity, microbiology, and genetics are at the heart of our understanding of the pathogenetic basis and pathophysiology of infectious diseases.

The host response to microbial pathogens is usually investigated by use of bulk methods, based on the implicit assumptions that the immune-cell populations studied are homogeneous and that the average differences obtained from bulk samples reflect what is happening at the level of individual cells [19]. However, bulk experiments average the responses of all cells in the sample and therefore, cannot differentiate between a weak response of a majority population and a strong response of a minority population. Moreover, single-cell experiments have shown that even populations of isogenic cells are functionally heterogeneous [20]. Single-cell studies are of particular interest in immunology, as immune responses are likely to be initiated by a limited number of cells (either of the innate-immune system that encounter a pathogen or by antigen-specific cells of the adaptive-immune system). Therefore, it is quite obvious that single-cell studies are required to grasp the full picture of the inherent diversity of the host responses, as well as a precise assessment of the molecular mechanisms of cell activation.

Flow cytometry and microscopy-based methods are among the most well-established single-cell technologies. With front-scatter (cell size) and side-scatter (cell granularity), flow cytometry can measure 19 parameters/cell in a high-throughput fashion [21]. However, most researchers use significantly less complex panels as the overlap of fluorochrome excitation, and emission spectra

limit the number of fluorochromes that can be used simultaneously without elaborate compensation [22]. Microscopy-based methods allow visualization of target location, trafficking, and the relation of the target cells to its environment [23]. However, microscopy-based methods are low throughput, labor intensive, limited by the number of parameters that can be analyzed simultaneously and are not conducive to automation.

New technologies are emerging to address some of the limitations of established single-cell approaches. Development of microfluidic technology has enabled researchers to perform highly detailed, high-throughput transcriptome profiling of single cells by use of multiplex qRT-PCR [24, 25]. Single-cell RNA sequencing-based methods are emerging to allow for the analysis of whole transcriptomes [26, 27]. This gives the opportunity to identify and reconstruct cell lineages without relying on previously established cellular markers [28, 29]. At the protein level, mass cytometry (CyTOF) is proving to be a very attractive alternative to polychromatic flow cytometry and is capable of measuring up to 44 parameters in parallel [30].

This article has 2 goals; first is to review how emerging single-cell technologies can contribute to improve our understanding of the host-defense response against microbial pathogens and the pathogenesis of infectious diseases, and second, we will discuss how single-cell investigations can lead to a better view of functional diversity among genetically identical immune cells.

## SINGLE-CELL DIVERSITY

Single-cell diversity is, in part, driven by the fluctuations of transcription, which is not a stable process but occurs in short bursts of activity, followed by periods of transcriptional silence (also known as “transcriptional pulsing”) [31]. Interestingly, bursting kinetics appear to be gene specific [32]. These random pulses of transcriptional activity contribute to the considerable heterogeneity of single cells that also occurs in monocultures [33, 34].

Factors that cause transcriptional or translational variation, often designated as noise, are classified as intrinsic or extrinsic factors [33, 34]. Variation in the biochemical process itself is classified as intrinsic, as it is gene specific [35]. Conversely, factors that influence gene expression indirectly, such as concentrations of transcription factors, are classified as extrinsic sources of noise. The majority of genes is represented by very little mRNA transcripts, which increases the impact of stochastic events [20, 36]. Cell division also increases heterogeneity when cellular content is distributed unequally during division between the 2 daughter cells [37]. Alternative splicing of pre-mRNA increases the functional diversity of proteins. Some cell types preferentially express just a single-spliced isoform, thereby increasing functional heterogeneity [27]. Furthermore, some genes, such as *IL2*, display a monoallelic expression pattern, where only 1 allele of a gene is actively transcribed [38]. According to recent data, monoallelic gene expression is a widespread phenomenon, implicated in up to 24% of genes expressed by mouse fibroblasts [39]. Similar findings were reported for human fibroblasts, in which biased allelic expression was detected for >75% of the investigated genes [40]. When the

allele of interest is heterozygous, monoallelic expression may lead to functionally distinct cells. Overall, numerous factors may render genetically identical cells functionally different.

## BULK VERSUS SINGLE-CELL ANALYSIS

Mean expression levels from bulk samples do not correlate well with the expression levels of single cells. This is because the pulsatile nature of single-cell gene expression results in a skewed or long-tailed distribution of mRNA expression levels that at least for some genes, correlates well with a log-normal distribution [41]. Data from Bengtsson and colleagues [41] on actin- $\beta$  mRNA expression profiles of single cells, isolated from the islands of Langerhans, are shown in **Fig. 1**. Expression levels follow an approximate Gaussian distribution on a log scale (upper), but with the use of a linear scale (lower; dotted line represents the mean), it becomes clear that the distribution is markedly skewed above and below the mean expression levels. Thus, it is impossible to infer reliably what occurs at a single-cell level from mean expression levels of heterogeneous cell populations [19]. Single-cell studies do not suffer from this averaging effect and therefore, can provide a clearer image of gene coexpression and regulatory networks when compared with data derived from bulk

studies [42]. In a recent study, Shalek et al. [27] showed that the response of single bone marrow-derived DCs to LPS is bimodal in terms of mRNA abundance and splicing patterns. Not all cells from a single sample respond to LPS, resulting in a bimodal expression pattern of numerous critical immune genes, including genes that are highly expressed at the population level. In a follow-up article, the same group of investigators demonstrated further that the bulk response to LPS is driven by a few early responder cells that activate neighboring cells [26]. Altogether, these studies paint an unexpectedly complex picture of the response to LPS, which is masked by bulk methods of analysis.

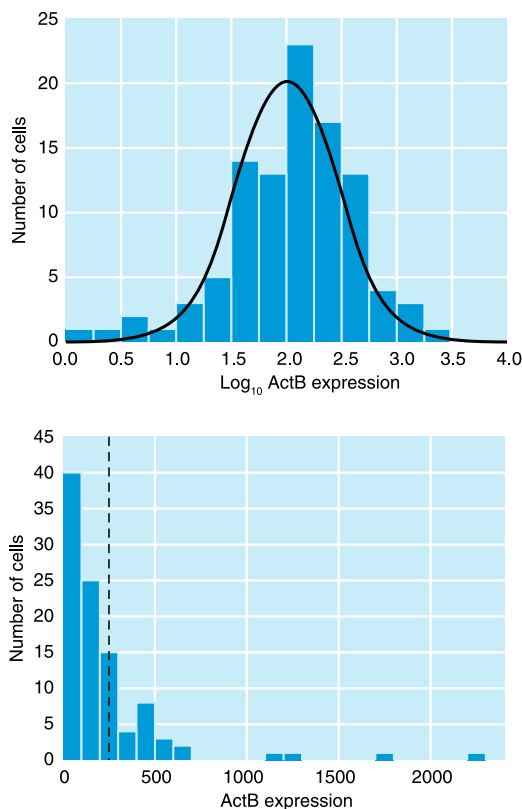
Given that stochastic effects have a great impact on transcription, single-cell gene expression is often labeled as noisy [35]. However, in contrast to what is normally classified as noise, this noise contains valuable information, offering critical insight into the mechanics of single-cell gene expression, and has functional consequences by increasing heterogeneity, which will be discussed in the next section.

## FUNCTIONAL HETEROGENEITY

As organisms become more complex, cells specialize to perform specific functions with higher efficiency. The immune system is a remarkable example of such functional specialization. It consists of an ever-expanding set of recognized subpopulations, as demonstrated by the recent discovery of innate lymphoid cells [43]. Likewise, recent work has led to the reclassification of human monocytes into 3 separate subpopulations of cells known as classic ( $CD14^{++}/CD16^{-}$ ), intermediate ( $CD14^{++}/CD16^{+}$ ), and nonclassical ( $CD14^{+}/CD16^{++}$ ) monocytes [44]. These subpopulations are thought to share a common lineage developmental path as the intermediate monocyte population increases before the nonclassical population during the course of an infection or after M-CSF treatment. However, gene-expression profiling revealed that intermediate monocytes have a distinct expression profile that seems incompatible with a simple transitional stage between the classic and nonclassical phenotypes [45, 46].

When cell populations are profiled at the single-cell level, even relatively well-defined populations may show considerable heterogeneity, a fact that is just started to be fully appreciated. This is illustrated by recent studies that use multiparametric mass cytometry. Based on the levels of 28 NK cell-surface markers studied in 22 human subjects, between 6000 and 30,000 distinct NK cell subpopulations were identified in each subject, for an estimated total of >100,000 distinct phenotypes [47]. As this classification is based on the expression of just 28 receptors, true phenotypic diversity is likely larger. To what extent the phenotypic heterogeneity of NK cells translates into functional diversity remains to be established.

The functional heterogeneity within apparently homogeneous populations and the large influence of stochastic events challenge the commonly held view that subpopulations represent distinct, predetermined developmental endpoints. Rather, this suggests that similar microenvironments lead to similar cells that can be loosely grouped together. Likewise, although useful in research and clinical settings, the notion that cellular populations can be defined accurately based on a few markers may be



**Figure 1. Single-cell gene expression of actin- $\beta$  (ActB) follows a log-normal distribution.** (Upper) Single-cell expression levels of ActB mRNA follow an approximate Gaussian distribution on a log scale. (Lower) The same data on a linear scale, revealing a highly skewed distribution. The dotted, vertical line corresponds to the mean expression level, illustrating that the mean expression level does not correlate well with the expression in the majority of the cells. Modified after Bengtsson et al. [41].

too restrictive. Starting from this assumption, researchers began to develop single-cell RNA sequencing-based methods coupled to algorithm clustering to identify cell types and lineages based on complete transcriptomes without having to rely on a limited number of predetermined markers [28, 29]. Such unbiased approaches may lead to improved understanding of cellular diversity and to the discovery of better markers to group similar cells (subpopulations). Next, we will discuss 2 emerging technologies that can be used to study the incredible diversity of immune cells: microfluidic single-cell gene expression analysis and mass cytometry.

## STRENGTHS AND LIMITATIONS OF MICROFLUIDIC SINGLE-CELL GENE EXPRESSION

Microfluidic devices excel in the manipulation of minute volumes, allowing a drastic size reduction and automation of classic methods [48]. An important strength is the ability to analyze primary cells from small samples, such as biopsies or lavages. This advantage was harnessed to develop a prognostic model of survival for colon cancer patients that performed better than standard pathologic grading, clearly demonstrating the clinical potential of the technology [24]. However, the major strength of microfluidics is the ability to perform highly multiplexed experiments. Thousands of qRT-PCR reactions can be run in parallel in a single experiment [49]. This strength comes with the limitation of increased experimental (see below) and analytic complexity. At present, there is a need for easy-to-use analytical tools, “standardized” statistical methods, and packages to analyze large and complex data sets generated by highly multiplexed, single-cell gene-expression experiments [50]. As an additional constraint, microfluidic single-cell gene expression experiments are expensive. This puts practical limitations on the maximum number of cells that can be analyzed, which in turn, limits the range of possible applications.

## CONSIDERATIONS FOR MICROFLUIDIC SINGLE-CELL GENE EXPRESSION EXPERIMENTS

Whereas microfluidic technology is very powerful, experimental design and setup are complex and require extensive validation.

This is mainly because the limited number of mRNA molecules/cell necessitates 2 rounds of amplification, during which initial transcript abundances need to be preserved [49]. This has several implications. First, primer efficiency needs to be very high at low mRNA abundances, which are typically not tested by the manufacturer and therefore, need to be validated manually. Second, efficiency must be similar for all primers to avoid unequal target gene amplification during preamplification. Third, primer competition and interactions need to be controlled for and avoided. Finally, an optimum number of preamplification cycles need to be determined so that low-abundance transcripts get amplified sufficiently while avoiding a leveling off in the amplification of high-abundance transcripts to prevent a bias of the initial transcript-abundance relationships.

## STRENGTHS AND LIMITATIONS OF MASS CYTOMETRY

Mass cytometry shares some principles with flow cytometry but differs on critical points (see also **Tables 1** and **2**). In both technologies, cells are labeled with conjugated antibodies. Whereas the antibodies are conjugated with fluorochromes in flow cytometry, in mass cytometry, the antibodies are conjugated with rare earth metal isotopes that are detected by a mass spectrometer [51]. This has major consequences. First, mass cytometry has no autofluorescence equivalent, as the isotopes used for labeling do not occur in biologic samples. Second, mass spectrometry panels generally need little to no compensation, as the instrument is able to detect adjacent isotopes specifically with very little overlap. Third, the multiplexing capability of mass cytometry is higher than flow cytometry, with experiments that use 44 parameters already published and a predicted possibility of analyzing 100 parameters in the near future [22, 30]. Fourth, the difference in signal intensity between the strongest and weakest isotopes is much less than the difference in signal intensity between the brightest and dimmest fluorochromes [52].

Limitations of mass cytometry compared with flow cytometry include throughput, which is 10–20 times lower (1000 cells/second) than the maximum throughput of common flow cytometers [22]. Additionally, only ~30% of all cells in a sample are analyzed versus nearly 100% for flow cytometry. Perhaps the most significant limitation is that cells are destroyed during analysis, so mass cytometry cannot be used to isolate cells for

**TABLE 1. Advantages of CyTOF compared with flow cytometry**

Property	Description
Background	Because the isotopes used to conjugate the antibodies do not occur in biologic samples, the background signal is very low. Consequently, CyTOF has no “autofluorescence” equivalent.
Compensation	Compensation is generally not required, as there is little overlap between adjacent channels. Minor adjustments might be required to correct for isotope oxidation, impurities of the conjugated isotope, or contamination.
Conjugate signal intensity	Whereas the signal intensity in CyTOF is lower than the signal intensity of the brightest fluorochromes for flow cytometry, the difference in signal intensity between the “brightest” and “dimmest” isotopes is much less than the difference in signal intensity between the brightest and dimmest fluorochromes.
Multiplexing and panel design	The multiplexing ability of mass cytometry is much higher than that of flow cytometry. In addition to the larger number of parameters, the design of large multiplex panels for CyTOF is less complex, as compensation is not as big of a factor as it is for large flow cytometry panels.



**TABLE 2. Disadvantages of CyTOF compared with flow cytometry**

Property	Description
Antibody availability	The selection of commercially available antibodies is still limited—both the number of clones and number of conjugates available for each clone. Therefore, for most panels, it will be required to conjugate manually purified antibodies by use of an antibody conjugation kit from the CyTOF manufacturer.
Cell sorting	Unlike flow cytometry, mass cytometry cannot be used to sort cells. This is because during acquisition, each cell is disintegrated so that it forms a cloud of ions. The isotopes used for conjugation are then detected within the ion cloud by time-of-flight mass spectrometry.
% of Sample analyzed	In contrast to flow cytometry, where close to 100% of cells in a sample is analyzed, in CyTOF, currently ~30% of the cells in a sample is analyzed; the remaining 70% is lost in transit to the detector.
Throughput	The maximum throughput for CyTOF is 1000 cells/second, which is 10–20 times lower than for common flow cytometers. Additionally, a significantly lower acquisition rate is often used to improve cell-event detection.

follow-up experiments. Finally, as in flow cytometry, the detection of a protein by mass cytometry requires a suitable antibody, which might prove troublesome for some targets.

### CROSS-VALIDATION OF MASS CYTOMETRY WITH FLOW CYTOMETRY

An important aspect of mass cytometry studies is cross-validation of antibody performance with flow cytometry. Cross-validation is essential as the conjugates and conjugation strategies potentially influence antibody specificity and affinity. Indeed, an antibody that performs well in flow cytometry experiments may have suboptimal performance in mass cytometry and vice versa. Therefore, antibodies generally have to be evaluated in parallel flow cytometry/mass cytometry experiments by comparing the fraction of positive cells in a given sample/subpopulation. An example of cross-validation is shown in **Fig. 2**. Cryopreserved PBMCs from a single healthy donor where stained with a panel, including anti-CD11c ( $x$ -axis) and anti-CD123 ( $y$ -axis), which can be used to identify the 2 main DC populations in blood (plasmacytoid DCs and myeloid or conventional DCs) [53]. An important difference between the flow and mass cytometry panels is that the negative populations are much more condensed in mass cytometry. This “crowding” around the axis is caused by the absence of an autofluorescence equivalent in mass cytometry. Besides the different appearance of the data and corresponding gate placement, the fraction of cells in each gate is similar, indicating that the antibody and conjugation procedure are compatible with mass cytometry.

### ANALYSIS OF HIGH-DIMENSIONAL MASS CYTOMETRY DATA

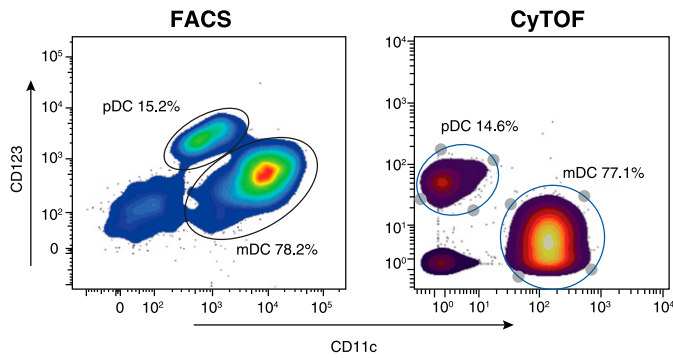
The data generated by mass cytometry experiments are similar to data generated by flow cytometry. However, the analysis of mass cytometry data with biaxial plots alone (as is common with flow cytometry data) is unfeasible as a result of the large number of parameters. To analyze a 34-parameter panel, 561 plots would be required [54]. Additionally, these plots would only show relations between 2 markers and not more complex relationships. *viSNE* and *SPADE* are 2 strategies to represent high-dimensional data in a 2-dimensional manner, while retaining as much information as possible [54, 55]. *viSNE* creates a sort of scatterplot, where each

cell is represented by a dot. The distance between the dots is determined by the similarity in expression of all measured parameters [55]. *SPADE* creates a minimum spanning tree based on density down-sampled events so that rare populations are retained in the process [54]. An example of *SPADE* analysis performed in our labs on cryopreserved PBMCs from a healthy donor is shown in **Fig. 3**. Cells were clustered into nodes based on the combined expression of CD3, CD4, CD7, CD8, CD14, CD16, CD19, CD33, CD56, CD123, and HLA-DR. These markers allow for a clear separation of the main PBMC populations. The size of the circles corresponds to the number of cells in the node. The overlaying of a color corresponding to the median expression level of a marker, in this case, CD4, makes it easy to see the expression of that marker in all populations simultaneously. The power of this approach is demonstrated by recent studies analyzing the response of PBMC subpopulations to diverse stimuli and inhibitors [22, 56].

### APPLICATIONS OF EMERGING SINGLE-CELL TECHNOLOGY

Besides single-cell qRT-PCR, microfluidic technology has been applied for RNA sequencing [26, 29], digital PCR [57], microRNA analysis [58], screening single nucleotide polymorphisms [59], single-cell haplotyping [60], measuring protein secretion [61], and chemotaxis [62]. Microfluidics technology is also used to develop diagnostic devices for use in low-resource settings, such as developing countries [62]. However, many applications of microfluidic technology have stayed at a proof-of-concept level [62, 63].

Mass cytometry has been applied to areas traditionally served by flow cytometry. Applications include cell-cycle studies [64], profiling of signaling via the TCR [65], phenotypic and functional analysis of CD8<sup>+</sup> T cells responses to nonspecific and antigen-specific stimulation [66], functional profiling of immune and drug responses across a hematopoietic continuum [67], and profiling of signaling cascades combined with a mass-tag cellular bar-coding approach to analyze drug responses in a high-throughput fashion [56] (see also **Table 3**). A bar-coding approach entails staining each sample with a unique combination of identification markers composed of chelated elemental isotopes (the bar-code). Samples can be pooled and analyzed in a single session, as analysis software can reconstruct the original samples based on the bar-code of each event. Just 7 markers are



**Figure 2. Cross-validation of mass cytometry antibodies with flow cytometry.** Cryopreserved PBMCs from a healthy donor were stained with a panel of antibodies, including anti-CD11c (x-axis) and anti-CD123 (y-axis). Events from the Lin2 (CD3, CD14, CD19, CD20, CD56)-negative subset are shown. (Left) Flow cytometry data. (Right) CyTOF data. pDC, Plasmacytoid DC; mDC, myeloid DC.

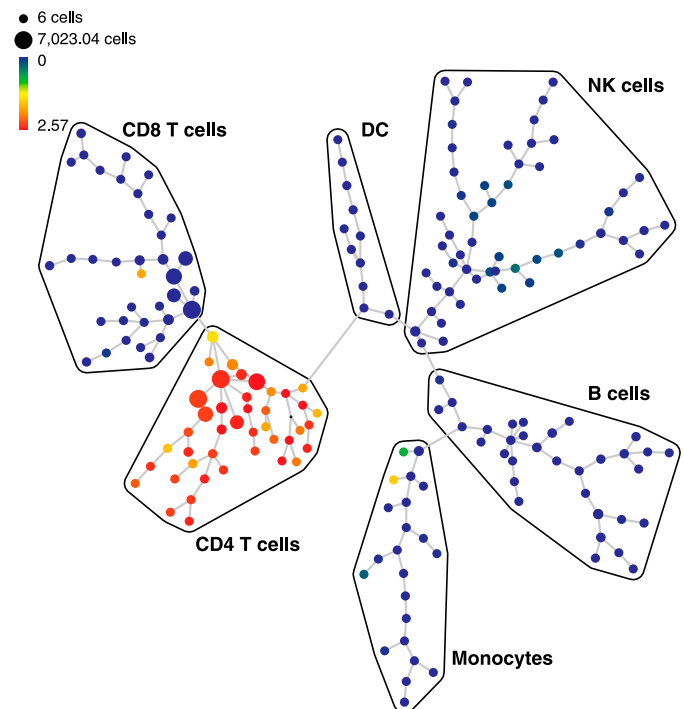
required to bar code a 96-well plate ( $2^7 = 128$  unique combinations), leaving more than enough markers free for detailed functional and phenotypic profiling. This unprecedented sample multiplexing enables the design of experiments that would be unfeasible by use of traditional methods. Bar-coding approaches can also be used to minimize bias introduced by slight variations in experimental procedures. This is demonstrated by a recent study, where the authors identified a strong correlation between signaling state of CD14<sup>+</sup> monocytes and recovery from partial hip arthroplasty [74]. As the authors used a bar-coding strategy, they could process all time-points from a single patient simultaneously, ensuring identical processing and eliminating inpatient bias related to sample handling.

Single-cell analysis can reveal features of biologic mechanisms that remain hidden by bulk experiments. The manner in which bulk measurements can be misleading is exemplified by a recent study on the early innate-immune response to RV [78], which is a major cause of severe diarrhea and death and has evolved mechanisms to evade the host innate-immune response [79]. To elucidate the mechanisms allowing RV to proliferate in IECs, Sen and colleagues [78] analyzed murine IECs from RV-infected mice. Bulk analyses unveiled that RV replicates with high efficiency in the small intestine, strongly inducing type I IFN and IFN-stimulated genes. However, the profiling of single-infected and uninfected “bystander” IECs from mice exposed to RV showed an absence of type I IFN induction or a reduction of type I IFN transcripts compared with control mice. This suggests that RV inhibits direct and indirect IFN induction in IECs. Moreover, it was shown that the majority of type I IFN is produced by cells from the hematopoietic compartment. Thus, whereas bulk measurements showed a strong induction of type I IFN after RV infection, single-cell profiling showed no type I induction in IECs. This study illustrates how bulk measurements can mask pathophysiological mechanisms and highlights the value of single-cell technology.

The “unmasking ability” of single-cell experiments may be particularly useful to study diseases for which current treatments are badly needed, such as sepsis. The prevailing concept has long been that sepsis is the result of a deregulated immune response to infection that is characterized by an overwhelming global

proinflammatory response causing life-threatening organ failures. Hence, the search for immunomodulatory treatment strategies aimed at counter-balancing excessive inflammatory reactions [80]. However, it is now clear that a “compensatory” anti-inflammatory response is induced simultaneously with the proinflammatory response, casting doubt on the validity of a universal proinflammatory only targeting approach [81]. Indeed, clinical trials aimed at counteracting the proinflammatory cascade in septic patients were mostly nonconclusive. Recently, leaders in the field have called for a rethinking of trial designs dealing with treatment strategies for severe infections [82–87]. Moreover, given the current debate, as to whether the genomic response models of critical illness accurately reflect critical illness in humans, it is clear that we have yet to capture the entire picture of sepsis pathogenesis [88, 89]. Single-cell studies are well suited to provide us with a better understanding of early events in the pathogenesis of human sepsis at the primary site of infection, as well as in the systemic circulation. Coupled with traditional (flow cytometry) or emerging (mass cytometry) immunophenotyping technologies, single-cell transcriptome profiling may prove to be extremely useful to explore regulatory immune pathways at the heart of the sepsis conundrum. Therefore, single-cell transcriptomics could lead the way to the development of novel sepsis therapies.

The ability of single-cell experiments to evaluate functional heterogeneity, regulatory pathways, and gene coexpression makes the technology appealing to study polarization,



**Figure 3. SPADE analysis of cellular hierarchies for the mapping of immune-cell lineages.** Minimum spanning tree created by SPADE showing the main populations of PBMCs. Cryopreserved PBMCs from a healthy donor were stained and analyzed with mass cytometry. The clustering of the cells is based on expression of CD3, CD4, CD7, CD8, CD14, CD16, CD19, CD33, CD56, CD123, and HLA-DR. The size of the circles corresponds to the number of cells in the node; the color overlay corresponds to the median CD4 expression level.

**TABLE 3. Selected CyTOF publications from the immunologic domain**

Subject	Cells	Main observation	Ref.
Signaling responses of human cells	Human bone marrow and PBMCs	Mass cytometry can perform highly multiplexed, detailed profiling in a high-throughput fashion, while also providing a systems-level view.	[67]
Cell-cycle analysis	HL-60, U937, NAL-6, and A20 cell lines; human PBMCs and bone marrow; mouse splenocytes	Cell-cycle analysis with CyTOF yields similar results as cell-cycle analysis with flow cytometry in both normal and cancer cells.	[64]
Mass-tag cellular bar-coding	K562 cell line, human PBMCs	The bar coding of samples enables highly multiplexed analysis for efficient screening experiments.	[56]
T cell epitope mapping	Human PBMCs and intraepithelial lymphocytes from proximal jejunum	Mass cytometric combinatorial MHC-tetramer staining allows highly multiplex profiling of T cell epitopes.	[68]
Mass cytometric analysis of small samples	Human PBMCs and skin-cell suspensions	Immune cell populations can be reproducibly identified in samples from 10,000 cells.	[69]
CD8 <sup>+</sup> T cell cytokine responses	Human PBMCs	CD8 <sup>+</sup> T cells produce incredibly diverse cytokine patterns after general or antigen-specific stimulation; MHC-tetramer staining.	[66]
T cell activation by antigen-specific stimuli	JKF6 and JY cell lines	Antigen density determines whether TCR engagement leads to T cell activation or anergy.	[65]
T cell remodeling by VZV	Human tonsil T cells	VZV infects naive and memory T cells and modulates cell phenotype, promoting skin homing and pathogenesis.	[30]
TCR signaling dynamics	Mouse lymph node and thymus cell suspensions	Signaling cascades after TCR ligation are nonlinear, which amplifies the effect of small, initial activation differences.	[70]
T cell subpopulations	Human PBMCs	CD161 defines specific functional subsets within CD4 <sup>+</sup> , CD8 <sup>+</sup> , and $\gamma\delta$ T cell subpopulations, which share innate-like characteristics.	[71]
Celiac disease	Human PBMCs and intraepithelial lymphocytes from intestinal biopsies	Gluten exposure in celiac patients induces gut-homing CD8 <sup>+</sup> $\alpha\beta$ , and $\gamma\delta$ T cells in peripheral blood with highly focused antigenic repertoires.	[72]
NK cell receptor diversity	Human PBMCs	Expression patterns of NK cell receptors are incredibly diverse, with an estimated number of phenotypes of 6000–30,000/person.	[47]
NK cell receptor expression	Human PBMCs	NK cells, T cells, B cells, and monocytes can be identified by their expression pattern of activating and inhibitory NK cell receptors.	[73]
Signaling dynamics in monocytes	Whole blood with erythrocyte lysis	Signaling state of monocytes correlates with clinical recovery after partial hip replacement.	[74]
Influenza vaccines	Human PBMCs	Influenza vaccines, based on degraded viral particles, activate monocytes and NK cells through Fc $\gamma$ R that depend on the presence of pre-existing IgG antibodies against influenza proteins in the serum.	[75]
HCV vaccines	Human PBMCs	A heterologous prime-boost vaccination strategy induces sustained, HCV-specific effector and memory T cells in healthy volunteers.	[76]
Mouse myeloid system	Mouse lung, liver, kidney, lymph nodes, thymus, and spleen-cell suspensions	High-dimensional analysis of the murine myeloid cell system enables identification of ambiguous and intermediate cell populations.	[77]

VZV, Varicella zoster virus; HCV, hepatitis C virus.

maturation, cell-fate decisions, and heterogeneity of subsets of immune cells. Macrophages are important immune regulators of homeostasis and are involved in the pathophysiology of numerous infectious and noninfectious diseases, such as autoimmune, welfare-associated diseases (obesity and cardiovascular diseases), and cancer [90]. Macrophages are often classified as belonging to the proinflammatory M1 phenotype (defined as classic activation) or to the anti-inflammatory M2 phenotype (defined as alternative activation) [91]. Skewed polarization toward a phenotype is assumed to play an important role in the pathogenesis and in the maintenance of many diseases [90]. Whereas the M1/M2 classification is useful as a guide, it is insufficient to capture the entire scope of macrophage heterogeneity [91]. A recent study in which human macrophages were exposed to 28 different stimulation conditions provided further evidence in favor of the existence of macrophage phenotypes beyond the classic M1/M2 dyad [92]. In an effort to increase our understanding of the factors involved in monocyte-to-macrophage differentiation, microfluidic single-cell technology was used to identify gene coexpression profiles during the differentiation of human monocytic THP-1 cells into macrophages [93]. Analysis of the temporal relation among expression levels of transcription factors led to the identification of key transcription factors regulating monocyte-to-macrophage polarization.

The studies described above demonstrate how single-cell experiments facilitate the identification of factors influencing cell-fate determination in immune cells. As many diseases are associated with skewed innate- and adaptive-immune responses, a better understanding of the cellular and molecular mechanisms underlying cell polarization and cell-fate decisions will undoubtedly help to improve our understanding of the pathogenetic basis of diseases and consequently, the identification of novel therapeutic targets.

The ability of single-cell studies to evaluate population heterogeneity can be harnessed to study the response to stimuli, such as LPS or PMA, but also to study the response to new vaccines and compare the effects of vaccine adjuvants [94]. Vaccines have greatly improved public health and helped to eliminate infectious diseases, such as smallpox [95]. Despite the successes of numerous vaccines, there remains a number of unmet vaccine needs against diseases, such as HIV infection, tuberculosis, and malaria, which continue to claim millions of lives worldwide each year [95]. The burden of dengue and the ongoing Ebola outbreak are other examples of unmet medical needs for vaccines [96, 97]. It is expected that single-cell gene-expression analysis will be of great additional value to analyze the response to vaccine vectors and adjuvants and to investigate why some vaccines offer protective immunity, whereas others do not. Additionally, the potential of emerging high-throughput, single-cell technologies is likely to be even greater in the analysis of low-abundance antigen-specific cells, as these rare populations will suffer most from the averaging effects of bulk studies.

## CONCLUDING REMARKS

Classic immunologic techniques based on the assessment of bulk populations of immune cells provide an imperfect global view

that greatly underestimates the constitutive and functional diversity of the immune system. This is a result of the averaging effect of combining the phenotypic and functional profile of single cells with all other cells in the sample. Thus, the contributions of minor populations of immune cells are masked by the overall biologic phenotypes, precluding a complete assessment of the full diversity of events and cellular responses that take place in any given biologic sample. Minority cell populations are of particular relevance to immunology, as the following: 1) the immune system features a high degree of cellular differentiation and functional specialization, and 2) immune responses are likely to be triggered by a limited number of cells that initiate the host-defense response upon encounter with a microbial pathogen. Emerging single-cell technologies have already shown their potential and value in immunologic studies. Their application will continue to expand as the techniques themselves and analysis tools evolve, becoming more affordable and accessible.

## AUTHORSHIP

All authors contributed to writing and editing the manuscript. J.H. performed the literary search. C.F. performed CyTOF experiments. J.H. and C.F. created the figures.

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## DISCLOSURES

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**KEY WORDS:**  
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