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Advancing systems immunology through data-driven statistical analysis

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Abstract

Systems biology provides an effective approach to decipher, predict, and ultimately manipulate the complex and inter-connected networks that regulate the immune system. Advances in high-throughput, multiplexed experimental techniques have increased the availability of proteomic and transcriptomic immunological datasets, and as a result, have also accelerated the development of new data-driven computational algorithms to extract biological insight from these data. This review highlights how data-driven statistical models have been used to characterize immune cell subsets and their functions, to map the signaling and intercellular networks that regulate immune responses, and to connect immune cell states to disease outcomes to generate hypotheses for novel therapeutic strategies. We focus on recent advances in evaluating immune cell responses following viral infection and in the tumor microenvironment, which hold promise for improving vaccines, antiviral and cancer immunotherapy.

Graphical abstract

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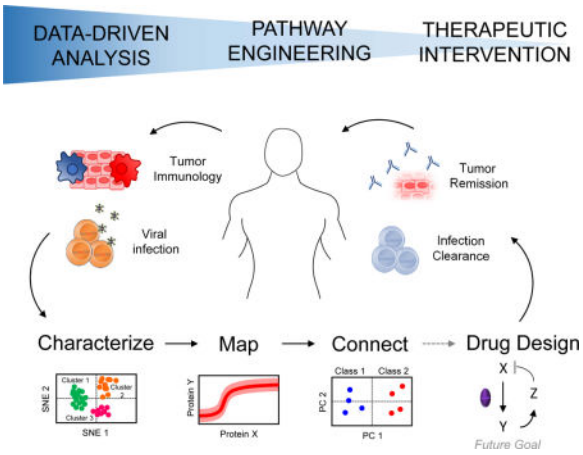

Multiplexed experimental technologies are accelerating data-driven systems immunology

Systems biology seeks to understand how elements of a protein or cellular network interact to produce diverse biological outcomes. Systems approaches that combine high-resolution multiplexed experimental measurements and computational analyses are especially advantageous when studying the immune system, a dynamic network of heterogeneous cell types and non-linear, interconnected signaling pathways [1]. To comprehensively characterize complex cellular behaviors, systems biology relies on multiplexed measurements of signaling and transcription in cell populations or single cells over time and in response to perturbations. Because phenotypic heterogeneity between immune cell types and within immune cell subsets is critical for immune function, the rapid increase in single-cell data collection in particular has catalyzed the growth of systems immunology [2–4].

Experimental techniques include RNA-sequencing, bead-based immunoassays, microwell assays [5,6], flow cytometry, and mass cytometry [7], and each approach has advantages and disadvantages. For example, mass cytometry (or cytometry by time-of-flight, CyTOF) enables multiplexed measurements of more than 40 proteins in thousands of single cell but requires pre-determined antibody panels [7]. In contrast, single-cell RNA-sequencing (scRNA-seq) measures thousands of transcripts for a more comprehensive and unbiased approach, but the measurements are more susceptible to experimental noise, particularly for low-abundance transcripts [2]. These differences must be considered when developing methods to generate biological insights from high-dimensional datasets.

Data-driven statistical algorithms for extracting information from large datasets (Table 1) have paralleled recent advances in multiplexed measurements, especially in single cells. Data-driven modeling uses mathematical analyses and computational algorithms to infer biological relationships. Computational data-driven approaches can be implemented even when knowledge of the underlying immune response mechanism is incomplete, as is often the case. The biological insights gained from data-driven modeling depend on the types of measurements available and the biological relationships being tested [8]. For example, data-driven models can be used to *characterize* immune cells by their associated phenotypes and

functions, *map* signaling network influences intracellular and extracellularly, or *connect* biological states with outcomes such as disease status (Figure 1). This review highlights recent examples of data-driven systems modeling that characterize, map, or connect components of the immune system, with a focus on applications in virus-host interactions and cancer immunotherapy.

Characterization of immune cell subsets and their functions

To characterize immune cell heterogeneity across functional subsets of the immune system, many researchers have interpreted high-dimensional protein measurements using dimensionality reduction techniques. *Newell et al.* measured surface markers and combinatorial cytokine expression of CD8+ T cells–critical effector cells of the adaptive immune system–by mass cytometry. They then used principal component analysis (PCA), a linear transformation of the data that maps cells onto lower dimensional axes of maximum covariance, to separate naïve, central memory, and effector memory CD8+ T cells and identified new functional features associated with these subtypes [9]. Amir et al. robustly separated distinct immune cell types in leukemic bone marrow by visualizing mass cytometry data with t-distributed stochastic neighbor embedding (t-SNE) [10], a non-linear dimensionality reduction algorithm that preserves local structure when mapping the high-dimensional space in two or three dimensions [11].

Functional heterogeneity also exists within a single immune cell type, as seen in natural killer (NK) cells and B cells, two populations critical for immune defense. To understand how both genetic and environmental components contribute to NK cell diversity, NK cells were isolated from monozygotic twins, characterized by 37-dimensional mass cytometry, and clustered using spanning-tree progression analysis of density-normalized events (SPADE) [12]. SPADE was a useful way to visualize how genetics and environment shape NK cell diversity because the algorithm emphasizes rare subpopulations, groups cells with similar phenotypes, and links clusters with weighted edges to identify relationships [13]. In a similar example, clustering of mass cytometry data tracking B-cell differentiation during rotavirus infection identified diverse mucosal trafficking phenotypes associated with humoral B-cell memory to the pathogen [14].

Given the growing number of dimensionality reduction and clustering techniques available to visualize and separate immune cell subsets, it is reasonable to ask how they perform relative to each other. *Wong et al.* characterized surface and functional markers of CD4+ T helper (T_H) cells, a subset of T cells that promote adaptive immunity by activating B cells and cytotoxic CD8+ T cells [15]. They found that t-SNE outperformed PCA for separating known T_H cell subsets; but isometric feature mapping (ISOMAP) [16], a method to visualize the relative similarities between subsets by mapping non-linear distances, was better at capturing how T_H cells progressed phenotypically over time [15].

A challenge in systems immunology is comparing newly discovered features of immune cell populations that were characterized in studies using different measurements and data-driven statistical methods. The robustness of these discoveries depends on the experimental method used and the number of dimensions measured: as more parameters are measured, clustering

and low-dimensionality projections sometimes become less stable due to increased data noise, and thus approaches for accurate, stable comparisons across high-dimensional studies are needed. To address this, *Spitzer et al.* developed a data-driven reference map, termed Scaffold map, by clustering single-cell data from murine bone marrow using force-directed graphs around “landmark nodes” of commonly recognized immune subsets. They then identified changes in immune composition across studies by comparing this reference map to Scaffold maps of different tissues and species across mass cytometry and archived flow cytometry data [17]. Such frameworks are essential for establishing a reference point to examine changes in immune composition associated with disease [18].

Some studies combine dimensionality reduction and data visualization with clustering to characterize distinct immune cell types within healthy and diseased microenvironments. For example, Tirosh et al. profiled malignant, immune, stromal and endothelial cells in metastatic melanoma patients using scRNA-seq, and used an algorithm called DBScan, which analyzes the density rather than the distance between data points to identify clusters of arbitrary shapes. They first used DBscan to cluster the data after dimensionality reduction using t-SNE, and then used PCA to identify subgroups within these clusters. Together, these methods characterized heterogeneous immune phenotypes and identified exhausted tumor-infiltrating T cells in melanoma tumors [19]. *Zheng et al.* combined t-SNE visualization with an unsupervised clustering algorithm called SC3, which returns robust and stable clusters by combining multiple clustering solutions to reach a consensus clustered dataset – an approach designed to address the instability of high-dimensional measurements. This analysis identified an enrichment of exhausted CD8+ T cells and inhibitory regulatory T cells in liver tumors relative to healthy liver, as well as potential regulatory genes enriched in these immune subsets [20]. These types of systems-level classifications will aid the design of novel cancer immunotherapies.

Mapping signaling network influences and network connectivity

Data-driven modeling can also map intracellular signaling and transcriptional networks or extracellular cell-cell interaction networks that regulate immune cell responses. Importantly, multiplexed measurements in single cells provide the thousands of observations typically required to mathematically extract network connectivity. To generate hypotheses for how dependencies between proteins change under different conditions, *Krishnaswamy et al.* developed conditional-density rescaled visualization (DREVI), a technique that emphasizes rare events (e.g., rare cells) by normalizing events based on conditional density, and then further analyzes mutual information between variables (e.g., proteins) in the rescaled data (DREMI) [21]. They applied these techniques to single-cell mass cytometry measurements of signaling network activation in naïve and antigen-exposed CD4+ T cells to show that DREVI effectively reconstructed signal response functions between T-cell signaling proteins, and DREMI quantified how signaling dependencies change in naïve versus antigen-exposed T cells [21]. In type 1 diabetic mice, DREVI analysis of T cell signaling revealed that small impairments in signaling activation proximal to the T cell receptor are amplified to produce larger defects at downstream signaling nodes [22].

A promising approach to map out immune signaling networks relies on taking high-dimensional measurements following targeted perturbations and then using data-driven approaches to identify network-level changes. For example, *Martins et al.* measured high-dimensional gene expression variation in monocytes after stimulation with cytokine perturbations representing different environmental contexts. By performing multiplexed qPCR on single cells and small cell groups, they were able to overcome "drop-out noise" for transcripts with lower abundance and calculate the propagation of cell-to-cell variation across multiple genes to uncover novel regulatory gene networks in macrophages that modulate noise to adapt to distinct environments [23]. Another study combined single-cell RNA-seq with CRISPR-pooled screens to measure how genetic perturbations affect transcriptome-wide regulatory circuits in single cells [24]. They identified unique cell phenotypes using dimensionality reduction and clustering with Phenograph [25], an unsupervised clustering algorithm that identifies nearest neighbors in high-dimensional space, and linked these phenotypes to their respective genotypes from the CRISPR screen. Similarly, *Xue et al.* used RNA-seq to measure macrophage transcriptomes after treatment with diverse stimuli, and used a combination of co-expression analysis, hierarchical clustering and self-organizing maps (SOM)–an unsupervised dimensionality reduction algorithm that projects and clusters data as a discrete lower-dimensional map–to identify stimulus-specific gene network programs [26].

To analyze how extracellular signaling networks regulated immune responses, our lab used Gaussian graphical modeling (GGM) to calculate partial correlations between proteins secreted by isolated macrophages, and reconstructed autocrine and paracrine signaling networks that drive the inflammatory stimulus response [27]. Another study characterized communication between the innate and adaptive immune systems by co-culturing monocytes and T cells and implementing self-organizing maps to uncover cytokines regulated by intercellular communication [28]. To dissect cell-to-cell communication across the entire immune system, *Rieckmann et al.* characterized human hematopoietic populations with mass spectrometry, and computed interaction network maps of "sender" and "receiver" cells by connecting the corresponding receptor-ligand expression levels between different cell populations [29]. Thus, data-driven modeling can identify networks and pathway connectivity in broad immunological settings.

Connecting immune cell states and composition with outcomes

Data-driven modeling can also be used to connect biological states to clinically important outcomes, such as disease progression or therapeutic response, as illustrated below with examples drawn from virus-host interactions and cancer immunotherapy.

Connecting immune cell states to various aspects of infection

Connecting systems-level changes in host cell signaling or gene expression patterns with the state or severity of viral infection can increase understanding of viral pathogenesis. Data-driven models have been used to connect alterations in neutrophil, NK cell, macrophage, and T cell function with increased susceptibility to viral infection [30–34]. Sen et al. developed a distance-based algorithm called SLIDE that quantifies the similarity between a virally-

infected cell and its most closely-related uninfected cell in multi-dimensional space [35]. *Cavrois et al.* used SLIDE to analyze differential surface marker levels between infected and uninfected memory T cells measured by mass cytometry and defined signatures of HIV-susceptible cells, termed 'predicted precursors' of infected cells [36].

Data-driven algorithms can also identify molecular or cellular signatures associated with virus manipulation of the host system. Using t-SNE visualization of cytokine and cell surface marker data measured by mass cytometry, *Hamlin et al.* showed that dengue virus alters the dynamics of cytokine production and primes the immune response to promote viremia rather than clearance [37]. Clustering analysis performed on whole proteome mass spectrometry profiles showed that HIV-infected patient CD4+ T cells on anti-retroviral therapy exhibited decreases in toll-like receptor 4 and type 1 interferon signaling relative to paired uninfected CD4+ T cell data [38]. To classify HIV+, T-cell depleted, and healthy PBMC samples, partial least squares discriminant analysis (PLS-DA)– which identifies linear combinations of variables to maximally separate known biological states –identified classification signatures based on measurements of 16 cytokines and chemokines, and then the underlying molecular changes could be associated with each group [39]. In a study from our lab, we measured phospho-signaling differences in uninfected and latent HIV-infected T cells and used PLS-DA to define signatures that predicted infection status and then identified changes in stress kinase signaling as a novel target for preferential infected-cell killing [40]. Comparing total immune cell composition in healthy and infected donors, SPADE and t-SNE analyses performed on 26-parameter mass cytometry data demonstrated that infected patients had a higher fraction of highly differentiated effector memory cells following HIV infection and ART treatment in comparison to healthy donors [41]. Overall, systems immunology studies of virus-host immune interactions have generated new hypotheses about mechanisms of viral pathogenesis and potential strategies to combat viral infection.

Connecting immune cell states to cancer

Data-driven approaches can also be used to generate immunological signatures from patient data that are predictive of clinical outcomes, a key goal in cancer immunology. *Chevrier et al.* used mass cytometry to profile tumors from renal carcinoma patients and found Phenograph-defined clusters of T cells and macrophages that correlated with progression-free survival [42]. A recent study by *Krieg et al.* used FlowSOM [43], a self-organizing map algorithm, and CellCnn [44], an algorithm that uses neural networks to detect rare cell populations associated with disease, to identify unique monocyte populations predictive of a clinical response to anti-PD-1 immunotherapy [45]. Together, these studies show that immune cells or system states can be connected in a predictive manner to biological and clinical outcomes.

Another goal is to identify immune populations correlated with disease progression that may become novel targets for immunotherapy. For example, patient-derived immune cells in tumor, non-involved lung tissue, and blood compartments were profiled using mass cytometry, and immune composition was defined using Phenograph clustering, which highlighted changes associated early lung adenocarcinoma progression and identified tumor-infiltrating myeloid cells as potential therapeutic targets [46]. *Spitzer et al.* used mass

cytometry and Scaffold maps to characterize immune composition across successful and failed immunotherapy treatments and connect peripheral CD4+ T cells to systemic immune responses required for tumor rejection [18]. Together, these systems-level studies uncovered key immune populations and potential therapeutic targets associated with disease.

Systems-level approaches also provide insights into mechanisms underlying cancer immunotherapy efficacy, which remain largely unknown. Infiltrating T cells from human and mouse melanomas were profiled by mass cytometry following treatment with immune checkpoint inhibitors anti-CTLA-4 and anti-PD-1, and condensed via clustering and PCA to reveal conserved and unique mechanisms for both immunotherapies [47]. In an example from our lab, we used microwell assays to characterize functional “secretome” changes in tumor-associated monocytes and macrophages (TAMs) following combination immunotherapy in melanoma [48]. Phenograph clustering of the secretome identified changes in TAM subsets, including the emergence of a novel TAM subset that secretes inflammatory cytokines with anti-tumor activity. Taken together, these studies demonstrate how systems-level profiling is uncovering new strategies to redirect the immune system for therapeutic benefit.

Conclusion

Systems immunology is poised for rapid growth. As the field matures, common analytical methods such as t-SNE have risen as consensus algorithms for non-linear single-cell data visualization, while new methods are being introduced to address data challenges that still limit biological interpretation. For example, new computational methods are being developed to address “drop-out noise” in single-cell RNA-seq data [49], to identify underlying data structures that are continuous and non-linear [50], and to speed up computation time [51]. Other challenges still to be addressed include integrating across different types of data sets: incorporating single-cell data with measurements collected in cell populations; and incorporating prior biological knowledge, such as time series and dose responses, to extract more biological insight from the same data set. In the next few years, we expect to see systems immunology studies move towards mechanistic discovery by combining data-driven approaches with hypothesis-driven modeling. By using data-driven modeling to generate a global view of the immune network and hypothesis-driven modeling to test specific mechanisms within the network, systems immunology will uncover new strategies to engineer the immune system and combat disease.

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Highlights (target for each: 85 characters with spaces)

- New experimental techniques provide high-content data of immune system function.
- Data-driven computational approaches enable new insights from immune cell data.
- Recent applications characterize immune cell subsets and map network influences.
- Connecting immune states to disease states will uncover new strategies for therapy.

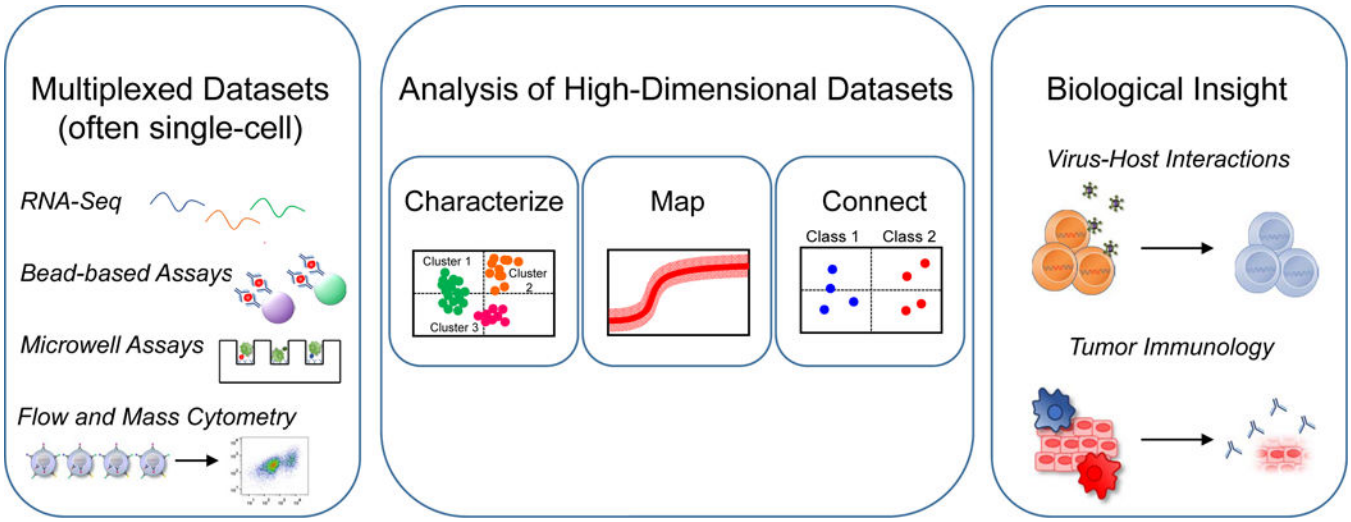

Figure 1.

Quantitative analyses shift the interpretation of large-scale systems datasets towards higher-order biological and mechanistic insights. The collection of high-dimensional datasets (left) can be computationally analyzed to *characterize* immune cells by their associated phenotypes and functions, *map* intracellular and extracellular signaling network influences, and *connect* biological states with outcomes (for example, disease status) (middle). These findings can be translated to identify new therapeutic targets to prime the immune system for antiviral and anti-tumor responses.

Table 1

Data-driven algorithms for high-dimensional analysis discussed in this review.

Method	Description	Ref.
PCA	A linear dimensionality reduction method that maps data into a new coordinate system that captures the covariation in the data	[52]
PLS-DA	A dimensionality reduction method that linearly links covarying signals to associated outcomes; can be used for classification and prediction	[53]
t-SNE	t-distributed stochastic neighbor embedding is a nonlinear dimensionality reduction method used for visualizing high-dimensional data in 2D or 3D	[10,11]
Self-organizing maps	A dimensionality reduction and unsupervised clustering method to visualize discrete populations on a map. FlowSOM is modified for flow- and mass- cytometry data.	[43,54]
SPADE	A clustering method that hierarchically orders changes in marker expression to depict groups in a minimally spanning tree	[12]
Scaffold Maps	A clustering method that visualizes cellular landscapes with pre-defined landmark populations	[17]
PhenoGraph	An unsupervised clustering method based on Louvain modularity often used to partition single-cell data into subsets	[25]
CellCnn	A clustering method based on convolutional neural networks that has been applied to detect rare cell populations associated with disease	[44]
ISOMAP	A trajectory visualization method that can be used to model cellular phenotypic progression based on the geodesic distances between cells	[16]
DREVI, DREMI	An inference method that applies conditional density estimation to visualize pairwise interactions and then calculates mutual information to score the strength of the interactions	[21]
SLIDE	An inference method that calculates differences between nearest neighbor cells to identify remodeled signaling pathways	[35]
Gaussian Graphical Modeling	A network inference method that quantifies partial correlations to define the dependence between variables.	[55]
DBScan	A clustering method that uses a density-based algorithm to discover clusters in high-dimensional space.	[56]
SC3	A clustering method that combined multiple clustering solutions to reach a consensus clustering of single-cell RNA-seq data	[57]