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***In vivo* systems biology approaches to chronic immune/inflammatory pathophysiology**

Alina Starchenko and Douglas A Lauffenburger

Systems biology offers an emphasis on integrative computational analysis of complex multi-component processes to enhance capability for predictive insights concerning operation of those processes. The immune system represents a prominent arena in which such processes are manifested for vital roles in physiology and pathology, encompassing dozens of cell types and hundreds of reciprocal interactions. Chronic, debilitating pathologies involving immune system dysregulation have become recognized as increasing in incidence over recent decades. While clinical consequences of immune dysregulation in such pathologies are well characterized, treatment options remain limited and focus on ameliorating symptoms. Because it is difficult to recapitulate more than a severely limited facet of the immune system *in vitro*, application of systems biology approaches to autoimmune and inflammatory *pathophysiology in vivo* has opened a new door toward discerning disease sub-groups and developing associated stratification strategies for patient treatment. In particular, early instances of these approaches have demonstrated advances in uncovering previously underappreciated dysregulation of signaling networks between immune system and tissue cells, raising promise for improving upon current therapeutic approaches.

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Introduction

Rather than being regulated by a set of exact parameters, each individual's immune system evolves under a set of loose biological guidelines, resulting in vast person-to-person variability [1,2]. This biological noise makes it difficult to separate normal variation from genuine disease drivers using conventional analyses [3]. Systems biology concepts and methods provide multi-variate approaches

to holistically analyze the larger interactive network of biological pathways and identify important players in inflammatory disease onset and progression.

The limitation of *ex vivo* and reductionist, univariate approaches in studies of inflammatory disease is the focus on previously defined immune cell populations and signaling interactions. Foundational work in immunology defines some subsets of immune cells as more or less important based solely on their surface markers through flow cytometry analysis. The cells within these subsets are often treated as homogenous and fixed in identity, which is untrue *in vivo* [4,5]. Immune cells are plastic [6,7], and thus their interactions generate dynamic signaling networks, among themselves and with cells of tissues within which they reside or into which they migrate [8]. Thus, a systems approach that considers the full spectrum of *in vivo* cell–cell signaling interactions is the only one that can hope to identify drug targets that will more probably make it through clinical trials.

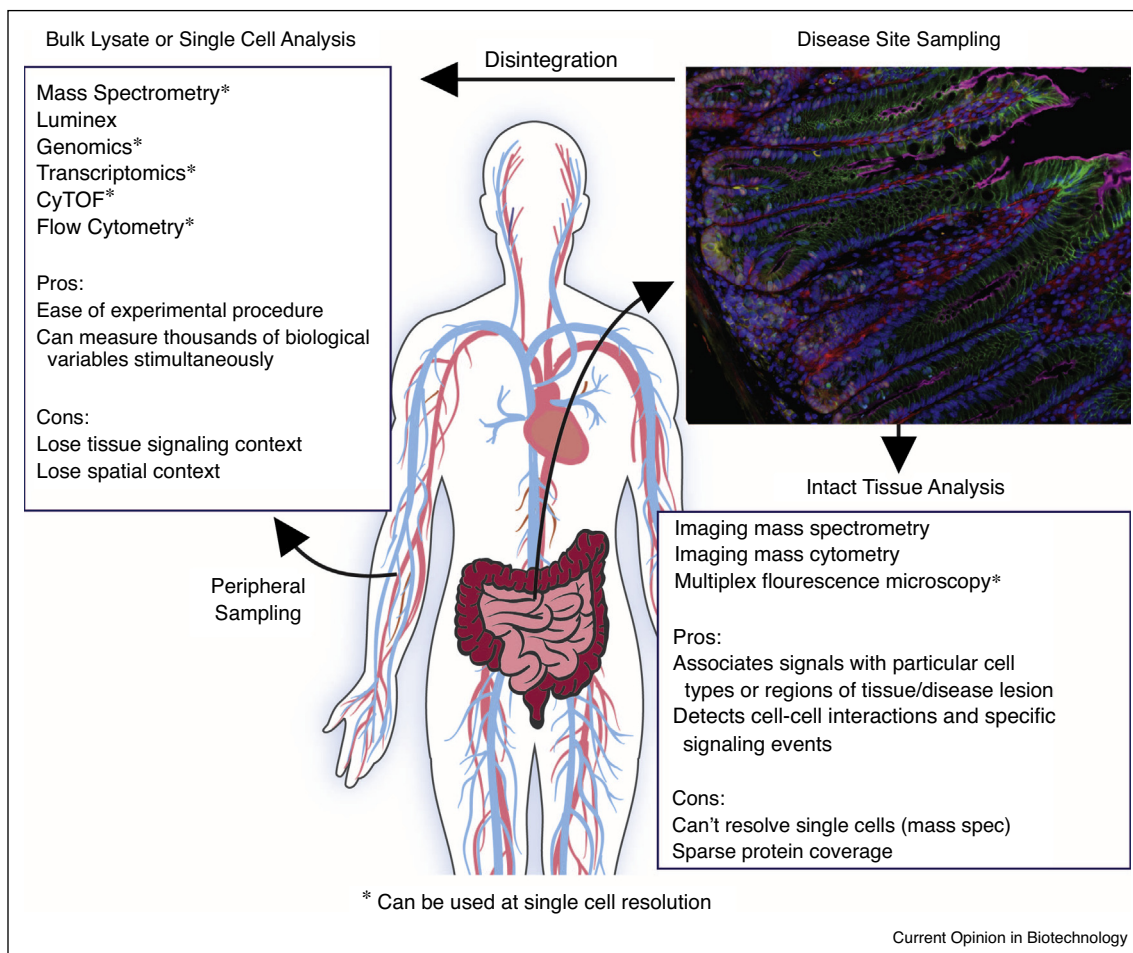
***In vivo* systems approaches identify biomarkers to better diagnose human disease**

A large effort has been put forth to identify biomarkers for diagnosis and stratification of patients for treatment. In the early days of genomic sequencing, GWAS (genome-wide association studies) promised to provide the information necessary to find populations at risk for a given disease, predict drug responsiveness, and identify disease subgroups. Although some genetic variations have been associated with inflammatory diseases such as Rheumatoid arthritis (RA), Inflammatory Bowel disease (IBD), Lupus Systemic Erythematosus (SLE) and others [9,10], these studies have not been as clinically transformative as hoped, with many patients displaying physiological pathologies but not possessing the associated gene variants. Further, GWAS have succeeded mostly in identifying correlated genes rather than targetable drivers [11].

Searching for peripheral biomarkers

Advances in transcriptomic and proteomic technologies (Figure 1) have allowed systems biologists to pursue biomarker identification using *in vivo* systems biology approaches [12,13,14,15–18,19,20,21]. Two categories of patient-derived *in vivo* samples are available for such research: fluids and tissues. Peripheral blood analysis is optimal due to its ease of integration into existing clinical procedures. Use of peripheral blood transcriptomics combined with a mixed modeling approach has led to models that stratify patients into sub-groups in SLE

Figure 1



Approaches to generating biological data for subsequent *in vivo* systems biology analysis.

and IBD. Such models identified several unrecognized subtypes of SLE and retrospectively predicted responses to existing treatments based on network parameters [14[•],19[•]]. The importance of measuring temporal fluctuations of *in vivo* signaling networks in autoimmune disease has also been highlighted and used to expand the IFN signature canonically thought to associate with SLE [19[•]].

The flexibility of systems analyses allows for combination of biological data with clinical parameters. This ability to combine heterogeneous data types allows for studies to identify molecular targets associated with particular clinical outcomes and to stratify patients based on either or both of these criteria [12,17]. Measurement of biological features in patient-derived fluids has identified subgroups within some inflammatory diseases, but has been largely unsuccessful in peripheral samples from IBD [22]. The latter highlights a potential limitation of sampling from a peripheral site: increased 'distance' between peripheral

blood and the primary disease site leads to dispersion of disease-specific biological material to the point where detection of the marker may fall to background levels.

Identification of biomarker states in the primary disease site

The dispersion problem can be solved through direct analysis of affected tissues (Figure 1). This approach is technically more challenging, but directly samples the signaling networks driving disease pathophysiology. Studies of intact tissue allow for analysis of interactions between immune cells and affected solid tissue cell types, whose destruction or malfunction results in patient symptoms. Signaling within tissues can be interrogated through either homogenization followed by -omics techniques, or disintegration into single cells followed by CyTOF [23,24], flow cytometry [25], or single cell -omics [26,27,28[•]]. A drawback of tissue dissociation is that it alters signaling of normally adherent cells within minutes [29]. Recent protocols have been developed to address

these issues, either through changes in the dissociation protocols [24] or through use of stochastic profiling, which is compatible with tissue processing techniques that minimize handling artifacts in the resulting pool of single cells [30]. Alternatively, imaging techniques that utilize tissue sections and retain spatial resolution of the sample can be harnessed to study cell–cell interactions [31–33]. These techniques have not yet been applied to studies of autoimmunity and inflammation, but their success in studies of pathologies in the same organ systems [34,35] make us confident in their future usefulness (Figure 2).

Application of *in vivo* systems biology to biomarker identification in inflammatory diseases has revealed their underlying molecular heterogeneity [12,14^{••},19[•],36–38]. These results may explain the failures of multiple clinical trials and the low response rate to existing targeted therapies. Further, they provide rationale for studying each disease driven by immune dysregulation as a set of molecularly different diseases with a similar pathophysiological outcome rather than as a single disease that can be treated with a single therapeutic approach.

Application focus: identifying drug targets

Current approaches to identify putative drug targets have not resulted in significant improvements to treatment of autoimmune and inflammatory diseases. Some, like SLE, have not seen major changes in treatment options in the past 50 years. Systems-based interrogation of *in vivo* signaling networks is beginning to predict useful drug targets by identifying previously unappreciated interactions in more physiologically relevant signaling networks.

Mapping signaling networks through perturbations *in vivo*

Historically, *in vivo* systems biology originated in signaling studies performed in murine models of inflammatory signaling. Early work used a multi-variate modeling approach to deconvolute the effects of TNF- α , a ligand known to stimulate both survival and apoptosis in a receptor-specific fashion [39]. The results of these early studies were astounding: not only was there a dose-dependent temporal control over cell response to TNF- α , but different regions of the intestine responded differently to the ligand [40,41]. Follow up studies utilized a similar approach to identify a set of kinase inhibitors to modulate these responses *in vivo* [42^{••}]. These studies demonstrate the dynamic and adaptive nature of signaling networks that is lost outside the *in vivo* context.

Signaling studies with human tissue

Mouse signaling networks do not generally translate directly to human ones [3,43–45]. Thus, some studies have focused on perturbation experiments in patient-derived tissues *ex vivo*. Multivariate analysis of signaling

in patient-derived tissues in RA has identified defects in B-cell signaling through defective cytokine production [46[•]] and determined how cytokine mis-expression contributes to disease progression through activation of synovial fibroblasts [13^{••}]. The caveat with these studies is that primary cells grown *in vitro* for even a short time differ from their *in vivo* counterparts, as determined through multivariate systems analysis of monocyte differentiation derived from human subjects [29]. Another methodology for gleaning *in vivo* information from publicly available patient and murine data represents how groups without access to mouse or patient samples can use systems approaches to generate or support hypotheses [47].

Mapping signaling networks in humans

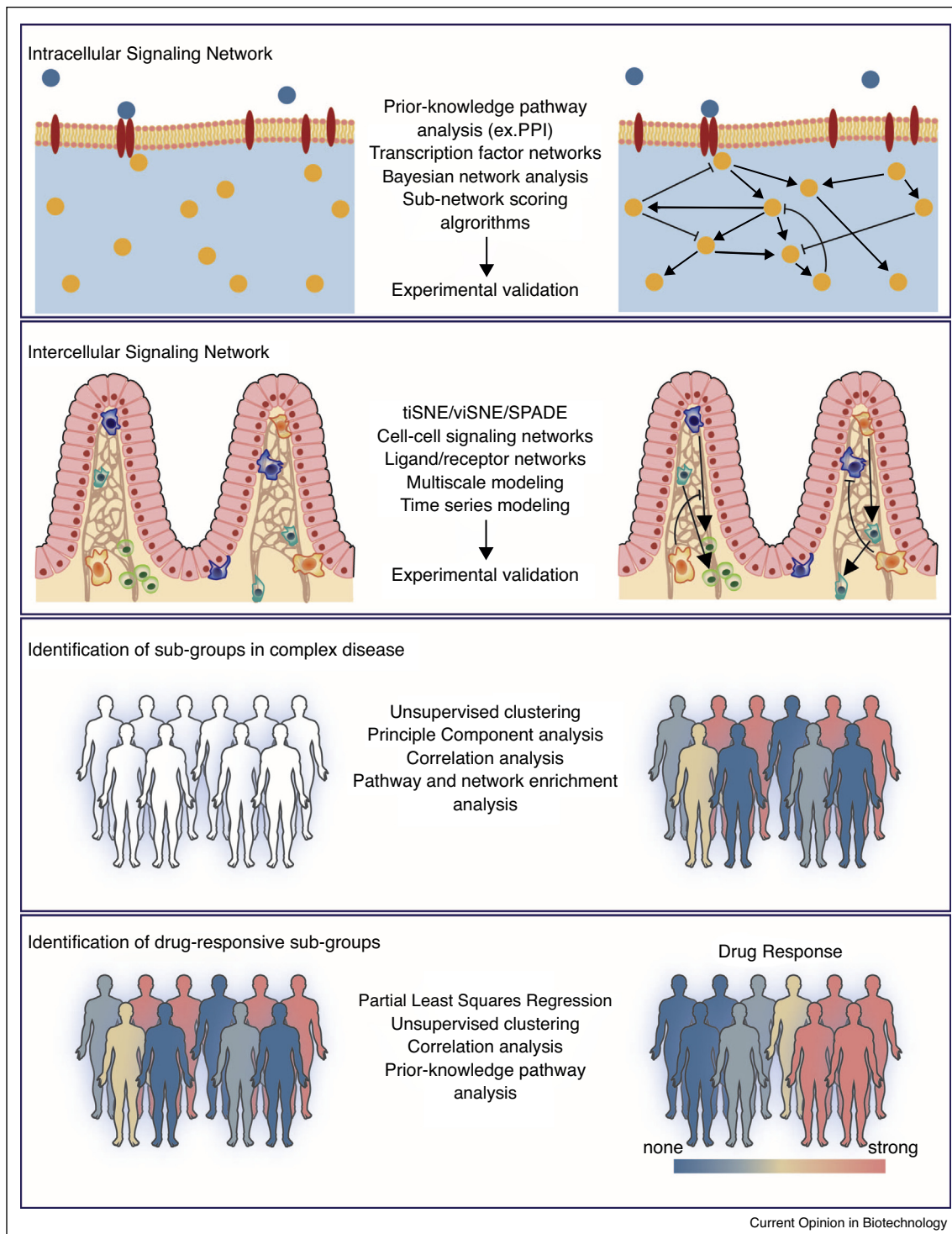
Two recent studies provide a blueprint and a rationale to probe human immune system signaling *in vivo*. In one, peripheral blood from Celiac patients exposed to a gluten challenge was sampled over time and analyzed through a combination of flow cytometry, CyTOF and single cell sequencing. The result identified coordinated responses between different T-cell types and suggests their involvement in the destruction of intestinal epithelial cells. This study also sheds light on how initial misdirection of CD4⁺ T-cells may cause effector T-cell mediated tissue destruction [48^{••}]. While treating human subjects with disease-activating stimuli is ethically challenging, applying this approach to patients prior to and after targeted drug treatment would provide invaluable insights into disease-driving signaling networks and may identify network components that associate with responsiveness or side effects, thereby bettering patient quality of life [48^{••},49].

Computational approaches: ‘So you want to use systems analyses . . .’

Many approaches exist to explore the structure of interconnected, multi-dimensional data such as that utilized by the studies discussed above. Three basic areas involved in the modeling of *in vivo* signaling networks include machine learning, graph theory and non-linear dynamics and chaos. We cannot hope to adequately cover these, but excellent reviews and perspectives can be found here [28[•],50,51]. Instead, we provide an example set of steps to begin exploring multivariate data [52].

Analysis often begins with hierarchical clustering as an exploratory method to provide a picture of the landscape of similarity and grouping of samples based on given measurements [53,54]. Machine learning multivariate projection approaches such as principle component analysis (PCA) and partial least squares (PLS) (PLS can be implemented as regression, for continuous responses, or discriminant analysis, for discrete states) can simultaneously provide insight into which measurements (protein, cytokine, cell type, and so on) or patterns of

Figure 2



Common systems biology approaches for particular problems in human health.

covarying measurements correlate with a given phenotype [55–57]. The resulting models can be built upon using prior knowledge pathway analysis, which draws from curated pathway databases like KEGG [58] and

GO to aid in extraction of biologically relevant interactions. Two commonly used tools for this extraction are Gene Set Enrichment Analysis (GSEA) and ALIGATOR [59,60]. Causal relationships can be determined using

Bayesian networks, which combine prior knowledge with data from the current experiment [61–63].

Together, these techniques can identify features of interest in a given network of biological interactions between immune cells and tissue, for example those that lead to an IBD lesion. Results from these studies must be properly cross-validated and require experimental follow-up, often implemented as an iterative process using computation to inform experiment and vice versa, leading to an understanding of not only how an initial disease-associated signaling networks differ from healthy ones, but how perturbation of a given target (cytokine, receptor, cell type) can alter the disease network to more closely resemble the healthy one. If the above looks daunting, note that one does not need all of these methods and that fairly simple approaches can be used to begin drawing interesting and testable observations from multivariate data.

Final thoughts: looking toward the future

Newly developed technologies are generating new types of data for systems analysis. Advances in mass spectrometry, imaging and sample processing promise to transform our ability to study cell–cell signaling *in vivo*. Mass spectrometry has been instrumental in providing data for systems analysis. Recent advances provide means to reduce required sample size for signaling studies [26], to detect protein interaction partners and to identify putative new phosphorylation sites [64]. Analysis of solid tissue has been hindered by its structural complexities. New protocols for disintegration of solid tissues and subsequent analysis by CyTOF will provide single cell resolution to solid tissue signaling networks [23,24], making the identification of disease-specific cell populations and the signaling events they participate in possible.

Advances in spatial visualization of proteins in a tissue through spatially oriented mass spectrometry provide an alternate solution to the solid tissue problem, and allow for direct observation of immune cell–target cell interactions [49,65–67]. Use of cyclic immunofluorescent approaches preserves the tissue sample for further analysis [34] thus theoretically allowing for multiple analyses from the same sample. A current disadvantage of the intact tissue approaches is the low number of measurements that can be generated (dozens) when compared to –omics approaches (thousands). However, these antibody-based can be coupled with antibodies specific not only to modifications such as phosphorylation, but also ones that detect protein complexes and conformational changes to add an extra layer of information [68]. Signaling by cells and within cells is complex and variable by tissue and cell type. The more layers of information gleaned about the network, the better it can be defined and the more likely we are to identify the features driving specific disease phenotypes.

Immunology-specific technical advances have begun to lower barriers to *in vivo* inflammation research. The substitution of traditional and limited gating strategies with viSNE and SPADE algorithms has allowed for identification of small cell populations and revealed elements of inflammatory network plasticity that are lost in methods employing univariate, non-single cell analysis [18,69–71]. The discovery that immune cells secrete and signal through exosomes also provides a new method of sampling peripheral blood by allowing for the concentration of biologically active particles, rich in protein and RNA components [72,73]. Plasma-derived exosomes are already being studied as putative biomarkers in several inflammatory pathologies. Whether human or murine, *in vivo* systems studies are expensive. Advances in micro-physiological ‘organ on a chip’ platforms that include immune cells as well as several interacting components show promise in reducing costs and making temporal analysis possible [74].

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