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Systems biology approaches to dissect mammalian innate immunity

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Summary

Advances in experimental tools have allowed for the systematic identification of components and biological processes as well quantification of their activities over time. Together with computational analysis, these measurement and perturbation technologies have given rise to the field of systems biology, which seeks to discover, analyze and model the interactions of physical components in a biological system. Although in its infancy, recent application of this approach has resulted in novel insights into the machinery that regulates and modifies innate immune cell functions. Here, we summarize contributions that have been made through the unbiased interrogation of the mammalian innate immune system, emphasizing the importance of integrating orthogonal datasets into models. To enable application of approaches more broadly, however, a concerted effort across the immunology community to develop reagent and tool platforms will be required.

Introduction

Evolution has given rise to a staggering scale and diversity of physical components underlying biological processes. Unlike traditional biological research that isolates and studies a small set of these components, the discipline of systems biology takes on this complexity by studying large numbers of elements of a biological process in parallel -- with the ultimate aim of generating comprehensive and quantitative models. The availability of complete genome sequences has spurred a series of technological breakthroughs in systematic, unbiased molecular profiling and perturbation. As powerful experimental and computational tools become widely available and affordable, systems biology will inevitably be applied to all biological systems – providing increasingly integrated and complete models of underlying molecular networks.

In this review, we highlight how systems biology has been used to analyze molecular mechanisms underlying mammalian innate immune responses to pathogens – focusing on measurement and perturbation studies and their integration to generate cellular network models -- and propose specific research programs that we believe will help the field move

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forward. The innate immune system appears to be present in every nucleated mammalian cell and functions to rapidly recognize infectious organisms (and other foreign entities), orchestrate the activation of the entire immune system and eliminate pathogens using a diverse arsenal of effector mechanisms. Recognition of pathogens relies on an array of sensors, including TLRs, RLRs and NLRs. Induction of immunity utilizes a large set of signaling components, especially secreted extracellular and membrane-bound molecules, and a unique system that presents antigens. Anti-microbial mechanisms are highly diverse in their mechanisms of action and include complement, defensins, inhibitors of translation and others. A systems-level analysis of innate immunity will shed light on host susceptibility and resistance to infections, mechanisms of vaccine action and the causes of diverse inflammatory disorders.

Using experimental perturbations and genetic variations for unbiased identification of functional elements

Progress in systems biology depends on having a robust suite of tools for perturbing genes in order demonstrate that a gene is involved in a process. Experimental perturbations provide the most definitive causal links between a gene and its functions. Methods to perturb genes include modifying DNA (e.g. engineering chromosomes, introduction of exogenous DNA for expressing genes), RNA (e.g. RNAi) or protein (e.g. antibodies, chemicals). Complementing this approach, natural genetic variation in humans or animals can also be used to identify causal factors that are associated with the outcome of innate immune responses.

Historically, saturation genetic perturbation screens in model organisms – such as the first ones to dissect the yeast cell cycle [1] or embryonic development of flies [2]– have revolutionized modern biology by identifying essential genes in many processes, and thus represent a central tool in any systems biology program. Indeed, genetically tractable model organisms like *Arabidopsis, Drosophila and C. elegans* have been used to identify evolutionarily conserved components of the innate immune system, including the Toll family of pathogen sensors [3], or more recently, entirely new pathways of gut innate immunity [4] or the role of calcium sensing protein kinases in transcriptional reprogramming of innate immune signaling [5]. Unbiased forward genetics screens have also become feasible in mice and have revealed multiple components of the mammalian innate immune system, including genes involved in sensing pathogens and resisting infection [6].

RNAi is possibly the most powerful systems biology tool for perturbing genes in mammalian cells. RNA libraries can be delivered as transfected dsRNA oligonucleotides or shRNAs expressed by viral vectors [7]. Early small-scale applications of this technology identified important regulators of the NF-κB [8]. Some genome-scale screens have been completed and revealed innate immune genes controlling necrotic cell death [9] and viral [10,11] and bacterial infections. A few studies integrated orthogonal datasets to identify components and their interactions in NFκB activation [12], TLR responses [13] and influenza-host interactions [14]. Finally, recent genome-wide pooled RNAi screens present a systematic, rapid and cost-effective method for finding critical genes (see example in cancer cells [15]). The availability of RNAi libraries will continue to inspire diverse innate immune screens while enabling sophisticated approaches such as gene-gene interaction screens to reveal network structures (see example in yeast, [16]). An important and complementary tool to RNAi is cDNA expression libraries that have led to the identification of genes based on their functional impact in specific assays (e.g. STING in DNA-sensing [17], MafB in IFN production [18]). Finally, an innovative and promising new method for disrupting gene function at the chromosomal level is the use of insertional mutagens in a haploid cell line

[19], a method that should enable saturation screens for genes involved in a myriad of cellular process.

Natural variation can also point to causal links between genes and traits or diseases, through classical positional cloning or genome-wide association (GWA) studies. For example, host genes affecting bacterial infections have been found in both mice and humans [20–23] and, in particular, Casanova and his colleagues have investigated the role of innate immune genes in human infectious diseases in depth [24]. When used to discover new genes, these approaches point to genes that are likely to be involved in host-pathogen and innate immune processes and need to be further validated. Outside of innate immunity, a systematic candidate gene approach discovered human gene alleles that cause respiratory chain disorders based on a multi-dimensional experimental and computational strategy that identified the complete mitochondrial proteome [25]. Having a relatively complete catalog of innate immune genes would similarly accelerate the analysis of rare genetic inflammatory disorders. In the future, with increasing statistical power from larger cohorts, it may also be possible to analyze genetic interactions that will help in the reconstruction of genetic networks controlling disease (especially when integrated with cellular innate immune networks).

Highly parallel molecular measurements

Monitoring the activities and physical modifications of molecular components -- such as transcript and protein levels, protein modification, enzyme-substrate relationships, protein-protein binding, transcription factor-promoter binding - is essential for revealing network topology and mechanistic interactions. For example: (1) Nucleic acid microarrays have enabled detection of the dominant coding transcripts in mammals while RNA sequencing is now enabling reconstruction of splice forms and discovery of non-coding RNAs; (2) quantitative mass spectrometry (e.g. SILAC) can be used to detect peptides representing thousands of proteins; (3) Peptide arrays can be used to find enzyme targets systematically; (4) HT yeast-2-hybrid screening can identify binary interactions. Datasets generated using these and other approaches can then be integrated using computational methods to formulate more comprehensive models of cellular processes (for a review of these approaches see [26]).

Microarrays have been used extensively to assess changes in mRNA levels of a host in response to specific pathogens or their components *ex vivo* or *in vivo*. For example, Zaslavsky et al studied the transcriptional response of *ex vivo* human monocyte-derived DCs to Newcastle disease virus [27], predicting a complex temporal cascade of transcription factors that reprograms the cell. Berry et al. profiled the blood of patients with TB to identify a neutrophil-driven interferon response associated with active but not latent disease [28]. If this signature is predictive of outcome, it could have significant impact on TB surveillance and treatment. Comparable studies for influenza have led to innate immune signatures associated with pathogenic strains [29,30] Another study identified innate immune signatures associated with CD8 or B cell immunogenicity in a highly effective yellow fever vaccine [31], providing ‘gold standard’ signatures that could be used to rapidly evaluate the potential efficacy of a new vaccine in patients. The routine use of nucleic acid microarrays in innate immunity has and will continue to generate extensive datasets that form a basis for mechanistic hypotheses, network reconstruction and biomarker discovery in health and disease.

A comprehensive understanding of innate immune components and their transformations will require monitoring changes not only in transcripts but also in protein abundance, structure. Quantitative mass spectrometry (MS) can be used to measure whole cell and

subcellular protein abundance, post-translational protein modifications, and macromolecular complexes associated with a protein of interest. For example, MS analysis of the lipidome and proteome of HCV-infected cells demonstrated a shift in energy metabolism and lipid profiles that may explain the impact of infection on cell survival and stress [32]. A comparison of two dendritic cell subtypes led Luber et al. to postulate and then validate that CD8+ and CD4+ DCs are differentially sensitive to RNA virus infection [33]. Analysis of the dynamic phosphoproteome of LPS-treated macrophages combined with a bioinformatic analysis generated an *in silico* kinase-substrate and transcription factor-promoter network [34]. Finally, to systematically identify DNA sensors, Jahn and colleagues identified proteins that physically associate with DNA and whose mRNA was known to induced by IFN-β. This approach yielded the cytosolic protein AIM2 that detects double-stranded DNA viruses, recruits the inflammasome and triggers IL-1β production [35]. Thus, MS can be a powerful unbiased approach to reveal a network's physical components as well as their modifications and interactions.

Integration of diverse molecular profiles and systematic perturbations into network models

By incorporating diverse information about the functions of single genes/proteins (from RNAi screens, protein-protein interaction networks and transcriptional response modules), systems biology attempts to gain a multi-dimensional view of a process and account for complex functions at a global level. While studies in yeast are far ahead of those in mammals, some recent efforts yielded significant insights into innate immunity. An early example of integrative systems biology used affinity purification and mass spectrometry to identify protein partners of 32 components of the TNF-α/NF-κB pathway, followed by computational analysis and directed functional RNAi-mediated perturbation to derive a functional interaction network [12]. More recently, Alexopoulos et al. analyzed 17 signaling intermediates and 50 secreted factors to determine how known innate immune pathways are rewired in transformed vs. primary hepatocytes [36]. In another report, Litvak and colleagues performed promoter motif analysis of transcriptional profiles in TLR4-stimulated macrophages and mathematically modeled and validated a feed-forward regulatory circuit – involving NF-κB, CEBP/δ and ATF3 -- to explain elevated cytokine production in response to persistent vs. transient bacterial stimulation [37]. This may protect the body from potentially harmful inflammation in response to spurious signals.

In a recent analysis, a consortium of laboratories used RNAi to reconstruct the network of 52 transcription factors predicted to be active during differentiation of THP-1 cells (a human monocytic cell line) based on motif activities derived from deep sequencing of transcriptional start sites (deepCAGE) [38]. Amit et al. devised a strategy to systematically knockdown 125 candidate transcription factors, chromatin modifiers, and RNA binding proteins (whose transcripts were regulated in response to TLR ligands) and then monitor the levels of ~100 representative TLR-responsive mRNAs [13] using a multiplex mRNA detection system. Their method enabled the construction of a network model consisting of 24 core regulators and 76 fine-tuners that help to explain how pathogen-sensing pathways achieve specificity. Together, these two studies demonstrated that a combinatorial and dynamic cascade of transcriptional regulators controls innate immune cell processes.

To characterize host and pathogen factors that participate in influenza-host interactions, our group recently developed a multi-dimensional approach which included the experimental generation of a human protein network that physically interacts with influenza proteins; decomposition of host cellular transcriptional responses to infection; functional assays to validate the role of 1745 cellular and viral factors in mediating interferon responses to infection or controlling viral replication; and computational analysis and modeling to

integrate the data and generate hypothesis that could then be further validated experimentally [14]. This study delineated the roles of hundreds of gene products that restrict or support influenza infection and modulate innate defenses. Although not focused on innate immunity, a study of HIV infection also integrated multiple datasets to derive subnetworks affecting distinct steps in HIV replication [39]. In addition while other studies dissect viral dependence on host factors, they focus on identifying cellular genes that facilitate viral replication rather than innate immune genes involved in restricting viral replication [40]. Finally, Kumar et al. use a genome-wide siRNA screen to coupled with microrray studies in THP-1 cells to elucidate networks of host factors that restrict TB infection [41]. Thus, integration of multiple experimental tools with computational methods is effective at revealing cellular networks at the host-pathogen interface.

The future of systems biology in the study of innate immunity

The ultimate goals of innate immune systems biology include: 1) reconstructing the molecular networks underlying innate immune processes; 2) unraveling the impact of natural variation in the human population; and 3) elucidating networks in patients with disease. However, systems biology is not sufficient to accomplish these goals; the resulting hypotheses and models must be further refined using traditional molecular and physiological approaches.

Realizing this vision will require a concerted effort to generate freely accessible reagents, highly reliable datasets and shared modeling and visualization tools that can then be used to produce integrated, predictive models. In addition, it also critical that experimental biologists and computational scientists collaborate closely to develop effective and innovative approaches in systems biology (for a review and examples of computational tools used to model dynamic innate immune responses, see: [42–46]).

Relevant datasets, computational tools and reagents are not necessarily unique to innate immunity. For example, comprehensive protein-protein interaction data, sub-cellular protein localization, transcription factor binding sites, catalogs of genetic polymorphisms are being generated and are useful for all biomedical research (see table 1 and [47]). However, a major hurdle is the lack of availability and access to genomic and genetic tools. In particular, validated RNAi libraries, genome wide ORFs and cDNA expression systems, comprehensive mouse knockout/transgenic/mutation libraries, as well as reliable antibodies and methods for monitoring gene expression at a robust and cost effective manner are absolutely essential (see Table 1). Given recent advances in these tools, it would be wise for the immunology community to take a page out of the yeast, worm and fly fields and generate, curate and centrally distribute a set of reagents (the ‘immunologist’s toolbox’) that enables to researchers to take on the systematic dissection of machinery that regulates innate immunity. The models resulting from these efforts will significantly impact medicine through improved identification of therapeutic targets and strategies, as well as elucidation of how innate immune networks differ across individuals and contribute to health and disease.

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Table 1
Public databases and resources for innate immunity research

Here we highlight key, publicly available, tools that facilitate systems biology approaches for the study of innate immunity. Note that some descriptions are quoted directly from the organization's website. There are also many commercial sources for similar information and reagents. In addition, there are non-commercial sources that are not listed (e.g. JAX, ATCC and others).

	Database	link	details
Immune Databases	Immunome	http://bioinf.uta.fi/Immunome/	a database for genes and proteins of the human immune system
	Innate Immunity Database	http://db.systemsbiology.net/IIDB	a repository of genomic annotations and experimental data for over 2000 genes associated with immune response behavior in the mouse genome, with particular emphasis on TLR genes.
	InnateDB	http://www.innatedb.ca/	a publicly available database of the genes, proteins, experimentally-verified interactions and signaling pathways involved in the innate immune response of humans and mice. The database captures the innate immunity interactome by integrating known interactions and pathways from major public databases together with manually-curated data into a centralised resource. The database can be mined as a knowledgebase or used with integrated bioinformatics and visualization tools provided at the site.
	Macrophages.com	http://www.macrophages.com	an online resource for

	Database	link	details
			those interested in macrophages and their role as major effector cells in innate and adaptive immunity. This website is designed to act as a centralised resource for the worldwide community of scientists interested in different aspects of macrophage biology
	Reference Database of Immune Cells (RefDIC)	http://refdic.rcai.riken.jp	an open-access database of quantitative mRNA and protein profiles specifically for immune cells and tissues.
	The Immunological Genome Project	http://www.immgen.org/index_content.html	a collaborative innitiative between immunologists and computational biologists who are generating a complete expression signatures of cells of the immune system in the mouse under various developemental and effector states.
	The Immunology Database and Analysis Portal (ImmPort)	https://www.immport.org	provides advanced information technology support in the production, analysis, archiving, and exchange of scientific data for the diverse community of life science researchers supported by NIAID/DAIT.
Pathways	Ingenuity Pathway Analysis (IPA)	http://www.ingenuity.com	a privately run company that provides self curated databases that allow researchers to explore, visualize, and analyze

	Database	link	details
			biological and chemical findings related to genes, proteins, and small molecules.
	Kyoto Encyclopedia of Genes and Genomes (KEGG)	http://www.genome.jp/kegg/	A bioinformatics resource that aims to provide a comprehensive representation of the cellular processes gained from genomic and molecular information. Includes protein interaction database, metabolic and signaling pathways, as well as chemical and drug databases.
	miRBASE	http://www.mirbase.org/	a searchable database of published miRNA sequences and annotation as well as aims to provide an extensive target prediction
	Pathguide	http://www.pathguide.org	contains information about 325 biological pathway related resources and molecular interaction related resources.
	Pathway Interaction Database	http://pid.nci.nih.gov	a database containing biomolecular interactions and cellular processes assembled into reliable human signaling pathways. Curently includes 114 human pathways manually curated by NCI-Nature and 322 human pathways imported from BioCarta/ Reactome
Human Genetics	National Human Genome Research Institute	http://www.genome.gov/gwastudies/	Provides a list of all GWAS

	Database	link	details
			publications (and data) that assay at least 100,000 single nucleotide polymorphisms (SNPs).
	The International HapMap Project	http://hapmap.ncbi.nlm.nih.gov/	a partnership of scientists and funding agencies from Canada, China, Japan, Nigeria, the United Kingdom and the United States to develop a public resource that will help researchers find genes associated with human disease and response to pharmaceuticals.
Protein-protein interaction databases	BioGRID	http://www.thebiogrid.org/	an online interaction repository with data compiled through comprehensive curation efforts. The current index is version searches 23,755 publications for 355,947 raw protein and genetic interactions from major model organism species. All interaction data are freely provided through the search index and available via download in a wide variety of standardized formats.
	Biomolecular Interaction Network Database (BIND)	http://bond.unleashedinformatics.com/	a comprehensive biomolecular data repository that stores curated descriptions of molecular complexes, pathways and biomolecular interactions.
	Database of Interacting Proteins (DIP)	http://dip.doe-mbi.ucla.edu/dip/Main.cgi	catalogs experimentally determined interactions between

	Database	link	details
			proteins. It combines information from a variety of sources to create a single, consistent set of protein-protein interactions.
	IntAct	http://www.ebi.ac.uk/intact/main.xhtml	provides a freely available, open source database system and analysis tools for protein interaction data. All interactions are derived from literature curation or direct user submissions and are freely available.
	MINT	http://mint.bio.uniroma2.it/mint/Welcome.do	focuses on experimentally verified protein-protein interactions mined from the scientific literature by expert curators. The full MINT dataset can be freely downloaded and viewed graphically within the 'MINT Viewer'.
	Pathogen Interaction Gateway (PIG)	http://molvis.vbi.vt.edu/pig/	a database dedicated to the study of host-pathogen PPIs. PIG provides a number of user interfaces for searching available data and tools for predicting interactions between host and pathogen proteins and between pathogen proteins.
	VirusMINT	http://mint.bio.uniroma2.it/virusmint/Welcome.do	aims at collecting and annotating in a structured format all the interactions between human and viral proteins and to integrate this information in

	Database	link	details
			the human protein interaction network.
Reagents	The I.M.A.G.E. Consortium	http://image.hudsonalpha.org/	Provide arrayed oligo dT-primed, directionally cloned plasmid cDNA libraries as part of several public EST projects, including clones from human, mouse, rat, zebrafish, *Fugu*, *Xenopus* (X. laevis and X. tropicalis), cow, and primate libraries.
	The International Knockout Mouse Consortium (IKMC)	http://www.knockoutmouse.org/	The members of the IKMC are working together to mutate all protein-coding genes in the mouse using a combination of gene trapping and gene targeting in C57BL/6 mouse embryonic stem (ES) cells.
	The Knockout Mouse Project (KOMP)	http://www.genome.gov/17515708	a trans-National Institutes of Health (NIH) initiative that aims to generate a comprehensive and public resource comprised of mice containing a null mutation in every gene in the mouse genome.
	Addgene	http://www.addgene.org	public plasmid repository for the research community.
	Mammalian Gene Collection (MGC)	http://mgc.nci.nih.gov/	Provides researchers with unrestricted access to sequence-validated full-length protein-coding (FL-CDS) cDNA clones for human, mouse, and rat genes.