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The Role of Systems Biology Approaches in Determining Molecular Signatures for the Development of More Effective Vaccines

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Abstract

Introduction: Emerging infectious diseases are a major threat to public health, and while vaccines have proven to be one of the most effective preventive measures for infectious diseases, we still do not have safe and effective vaccines against many human pathogens, and emerging diseases continually pose new threats. The purpose of this review is to discuss how the creation of vaccines for these new threats has been hindered by limitations in the current approach to vaccine development. Recent advances in high-throughput technologies have enabled scientists to apply systems biology approaches to collect and integrate increasingly large datasets that capture comprehensive biological changes induced by vaccines, and then decipher the complex immune response to those vaccines.

Areas covered: This review covers advances in these technologies and recent publications that describe systems biology approaches to understanding vaccine immune responses and to understanding the rational design of new vaccine candidates.

Expert opinion: Systems biology approaches to vaccine development provide novel information regarding both the immune response and the underlying mechanisms and can inform vaccine development.

Keywords

systems biology; vaccines; viral vaccines; vaccination; vaccine development; immunology; gene expression profiling; genomics; proteomics

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Disclosure Statement

Dr. Poland is the chair of a Safety Evaluation Committee for novel investigational vaccine trials being conducted by Merck Research Laboratories. Dr. Poland offers consultative advice on vaccine development to Merck & Co. Inc., Avianax, Adjuvance, Valneva, Medicago, Sanofi Pasteur, GlaxoSmithKline, Emergent Biosolutions, and Dynavax. Drs. Poland and Ovsyannikova hold patents related to vaccinia and measles peptide vaccines. Dr. Kennedy holds a patent related to vaccinia peptide vaccines. Dr. Kennedy has received funding from Merck Research Laboratories to study waning immunity to mumps vaccine. These activities have been reviewed by the Mayo Clinic Conflict of Interest Review Board and are conducted in compliance with Mayo Clinic Conflict of Interest policies. This research has been reviewed by the Mayo Clinic Conflict of Interest Review Board and was conducted in compliance with Mayo Clinic Conflict of Interest policies.

1. Introduction

Vaccines represent one of the greatest achievements of modern medicine [1]. Vaccines provide immunity against lethal and non-lethal diseases and have saved millions of lives. Despite recent breakthroughs in immunology, knowledge gaps still exist in terms of understanding the complex, tightly choreographed chain of events that allow the myriad cells, tissues, receptor-ligand pairs, soluble mediators, and other components of the immune system to work together in an integrated and sequential fashion to create protective immunity. The empirical design of vaccines using an “isolate, inactivate, and inject” paradigm and the “one size and dose fits all” approach to vaccine administration have been successful and significantly improved public health [2]. By using these approaches, lethal and disabling diseases such as polio, smallpox and tetanus, which once were major global threats to public health, are now under control or eradicated. Unfortunately, these vaccine approaches have limitations and have not been successful in developing vaccines for current public health threats, such as HIV, malaria, Zika, or tuberculosis. Recent advances in biology, virology, bacteriology, and immunology have demonstrated that an immune response to a vaccine is influenced by numerous factors, including age, gender, race, the quantity and quality of antigens, the route of immunization, number of administered doses, environment, nutrition, the microbiome, and metabolic activity, among others [3–5]. The tremendous inter-individual variations in vaccine response have hampered efforts to develop a set of defined rules governing the behavior of the immune system. As the population ages and develops immune deficiencies, the “one size fits all” approach becomes less reliable. For example, hepatitis B vaccine (HBV) is generally given as a series of three doses for adults; however, data suggest that up to 40% of the young adult population develops protective immunity after only one to two doses, while some individuals need multiple doses (i.e., six or more) to achieve protective immunity [6, 7]. More challenging is the development of vaccines against hypervariable viruses (e.g., influenza) and complex pathogens, such as HIV, dengue virus, plasmodium falciparum, leishmanial, and trypanosome cruzi. Past efforts have led to expensive clinical efficacy and safety trials that have generally not resulted in high-efficacy licensed vaccines [8]. Identification of the immunogenic antigens offering long-term protection against these complex pathogens is a major challenge in vaccine research. There is also a struggle to quickly translate these findings to newly emerging infectious threats. The trial-and-error process to develop vaccines has heavily relied on animal models, followed by clinical trials in humans. Animal models (most commonly mouse models), though useful for understanding the cellular and molecular mechanisms of immune responses to vaccines, have not successfully predicted the results of human vaccines. Possible explanations for this low predictive capability are lower life span, differential pathogen, susceptibility and exposure to different types of pathogens and microbiota, and genetic variations. Over their much longer lifespan, humans are exposed to a wider range of pathogens and microbiota compared to laboratory animals that are kept in pathogen-free (if not sterile) environments throughout their much shorter lifespans. The human microbiota consists of trillions of symbiotic microbes in each person. The gut microbiome interacts with and heavily influences the development and function of the immune system, which is a process that begins as early as the neonatal stage of life [9]. Recent data also emphasize the microbiota’s role on vaccine responses [10]. Humans are rarely homozygous for many

genes, while inbred mice are homozygous across most of their genome. Even using primates as an animal model to predict success of HIV vaccine has been unreliable [11]. While animal models have their uses, humans are the best model for truly understanding human immunity.

The immune system is a complex network consisting of organs and tissues, several hundred cell types, and thousands of soluble and cell-bound mediators that communicate and interact on different levels throughout the body to create protective immunity against infectious organisms. This complex communication includes, but is not limited to, biochemical intra-cellular and inter-cellular networks, as well as cellular inter- and intra-organ trafficking and dense networks. To understand the behavior of this complex system and predict its response to a vaccine, it is necessary to interrogate its parts together holistically rather than separately. The function and behavior of each part is context dependent and wired to many other parts of the immune system. To get a global, holistic view of how the immune system interacts with other biological systems and how these interactions govern response to a vaccine, immunologists and vaccinologists have turned to systems biology approaches. Systems biology is an interdisciplinary approach that integrates many scientific disciplines, such as biology, microbiology, chemistry, bioinformatics, physics, computational modeling, statistical analysis, and computer science to understand and predict behavior of the whole body as a system under varying conditions. Rather than a replacement of the traditional reductionist approach, systems biology should be seen as a complementary approach. There are several key elements in these systems biology approaches. First is the use of high-dimensional, “omics” technologies that allow us to interrogate, in an unbiased and comprehensive manner, biological responses whether they exist at the gene, mRNA, protein, metabolite, or cellular level. The second component is the use of advanced analytical routines and computational modeling approaches that allow us to investigate the incredibly large datasets that result from these omic technologies. Finally, the end goal is to develop novel biological insights and to create predictive models of the complex behavior observed after vaccination. Ideally, this leads to a more complete understanding of individual components, their interactions, and the emergent properties of the system as a whole (Figure 1). This holistic incorporation of scientific disciplines can provide insight into how the immune response works and identify the crucial targets that the immune system must recognize in order to develop a protective response to a pathogen. Vaccine immunogenicity is largely determined by the extent to which vaccination leads to a response that mimics an actual infection. Thus, a more complete understanding of how a response develops is critical for the rationale, design, and development of new vaccines and to elucidate ways to improve existing vaccines.

2. High-throughput technologies for immune characterization

The ability to comprehensively assess biological activity is an integral part of systems biology, and recent advances in high-throughput technologies have enabled us to evaluate immune function at a breadth and depth never before possible. In this review, a number of high-throughput technologies, with a focus on those technologies measuring biological features important for vaccine development, will be discussed. The review begins by outlining these technologies and their application in vaccine development. Approaches to

handle these data and how to integrate findings from systems biology studies into novel vaccine development will also be discussed. Finally, this review will highlight the challenges that are facing systems biology approaches to vaccine development.

Since the objective of systems biology is to design models of the immune system, it is essential to evaluate changes caused by a vaccine as comprehensively as possible. “Omics technologies” allow investigators to explore the entire proteome, genome, epigenome, metabolome, and other -omes in a systematic, simultaneous, and quantitative manner and are ideal experimental tools for this approach.

2.1. Microarray and Next Generation Sequencing

Following its invention in the 1990s, gene expression microarray became the assay of choice for large-scale genomic studies [12]. This technology remains a popular approach for transcript profiling because it is an established, inexpensive technology utilized to generate gene expression data sets. The RNA is extracted from the cells treated under different experimental conditions and converted to cDNA or cRNA [13]. The cDNA or cRNA is then labeled by a variety of tags including fluorescent, chemiluminescent, and radioisotope. The labeled cDNA or cRNA is hybridized with spots containing identical DNA strands (probes) that are mounted on a glass slide as (microarray). The probes at each spot represent a gene. The microarray is scanned or imaged to obtain the pattern of hybridization and to define the relative expression level of the genes in the sample [12, 14]. DNA microarrays have been used to identify genomic signatures in patients with severe influenza A [15–17], rhinovirus, and respiratory syncytial virus infection [17]. These genomic signatures are useful for the early diagnosis of influenza and classification of symptomatic individuals after influenza challenge, which may also help to expedite future vaccine-efficacy studies. DNA microarrays were also used in the following ways: to identify gene expression signatures in malaria [18] and dengue virus infections, [19] as well as in HIV patients [20]; to study variations in both innate and adaptive immune response to vaccines at different timepoints (e.g., influenza vaccines [21–23] and meningococcus [24] vaccines); and to study genes associated with HLA class I antigen presentation in recipients of adjuvanted RTS,S malaria vaccine [25]. Microarrays have been used to probe transcriptomic responses to the yellow fever YF-17D vaccine and identified gene-expression markers that predict YF-17D-specific cellular and humoral immune responses with 90% accuracy [26]; however, microarray technology has limitations, such as background hybridization, which limits the accuracy of expression measurements. Microarrays are effective for quantifying the expression of known genes and transcripts, but they do not detect changes in previously unidentified genes or transcripts.

Next-generation sequencing (NGS) technology enables the direct sequencing of DNA or RNA in a massively parallel fashion, allowing for millions of base pairs to be quickly and easily sequenced. RNA-seq does not require the probe-selection step required for the microarray approach; therefore, it avoids the inherent biases in that process.

RNA-seq has been used in the following ways: to monitor AS03-adjuvanted A/H5N1 avian influenza vaccine responses [27]; to identify transcriptomic signatures and key marker genes for development of vaccine-induced cellular and humoral immunity in older adults after

seasonal influenza vaccination [28]; to examine the role of HLA polymorphisms [26, 29]; and to study innate and adaptive responses to inactivated (TIV) or live attenuated (LAIV) influenza vaccines [23]. Importantly, this technology has allowed investigators to uncover a strong correlation between prior influenza vaccination history and migrating plasma cells and myeloid dendritic cell response one day after vaccination [22]. RNA-seq is also being used to investigate the mechanisms driving vaccine responses to Herpes zoster (shingles) in both young and old cohorts [30]. Using RNA-seq in a systems vaccinology approach to study Ebola vaccine rVSV-ZEBOV responses, Rechtién *et al.* discovered early innate immune signatures correlated with peak antibody titers [31].

As demonstrated by these studies, RNA-seq provides important insights into transcriptomic activity associated with successful vaccine responses. In some studies, these transcriptomic signatures are correlated with immune effector mechanisms that cannot be easily measured. This allows us to develop better correlates of protection that can be easily monitored in the blood, potentially eliminating the need for bone marrow biopsies to evaluate plasma cells, or lymph node biopsies for T_{FH} cells, or taking tissue samples to evaluate CD8⁺ T cell activity in its true biological context. These studies have also identified pathways activated by adjuvants—allowing us to understand mechanisms of adjuvant activity. This information could guide the development and use of specific assays to assess vaccine immunogenicity or to inform adjuvant selection so that critical innate signaling pathways are appropriately activated in response to the vaccine.

2.2. Next Generation Sequencing for Ig and TCR repertoire analysis

B cell and T cell specificity is determined by the immunoglobulin (Ig) and T cell receptor (TCR) surface proteins that are coded for by a large set of genes produced by imprecise somatic gene recombination. The ability to generate millions of sequencing reads is now routine and allows us to capture the tremendous diversity of the T and B cell receptors and to study clonal expansion at the genomic level after vaccination. The first step in this process is the formation of a cDNA library, which is then used to synthesize new DNA fragments. NGS can sequence billions of DNA fragments from a single sample through parallel sequencing. This facilitates high-throughput sequencing, which allows whole-genome sequencing in less than a day.

NGS has been used to characterize human B cell repertoire diversity in response to influenza vaccination [32, 33]. Investigators found that, compared to younger subjects, the Ig repertoire in older individuals receiving the vaccine was far less diverse, suggesting an age-related narrowing of B cell response [34]. The use of NGS for TCR/BCR sequence analysis has led to the following events: the interesting observation of convergent antibody rearrangements specific to influenza antigens, which indicates pathogens can induce specific Ig gene rearrangements [32] and could potentially serve as molecular signatures of infection history; the discovery of virus-specific memory CD4⁺ T cells in individuals who had never been exposed to the virus, which has significant implications for immunity to new pathogens and the influence of hygienic versus pathogen-rich environments [35]; and the discovery of persistent, EBV-specific clonal expansions that were unrelated to vaccine responses—a finding that may allow us to differentiate human B cell responses to chronic infections

versus acute infections (or vaccinations) [36]. TCR and BCR diversity can serve as a molecular signature describing the nature and quality of the immune response.

A new emerging application for NGS is sequencing of antibody and TCR repertoires on the single-cell level in response to vaccine [37, 38]. In this approach, single T or plasma blast cells are separated into distinct wells where they are lysed and Ig and TCR chains are amplified and sequenced. A unique oligonucleotide “barcode” is ligated to all products from a single cell. After their amplification, products from different cells are combined and sequenced together. The barcodes enable regrouping of the information collected from each single cell; therefore, a complete TCR or Ig sequence from each single T cell or plasma blast is obtained that be used to reconstruct TCR and Ig and determine specificity of Ig and TCR and also affinity of the antibody [38, 39]. Overall, high-resolution characterization of Ig and TCR repertoire by NGS can show changes that occur following vaccination and measure immunogenicity and specificity of vaccine. It can also be used to compare the vaccine response to the infection response and identify differences that may affect long-term immunity. TCR and Ig sequences can be characterized on an individual T or B cell basis, making it possible to reconstruct the antigens targeted by those sequences [38, 40, 41]. Prediction of epitope target by analysis of Ig and TCR repertoire can be used to validate antigens selected through reverse-vaccinology approaches or to identify regions of the pathogen genome that are hot spots for immunologic recognition [42, 43].

A new promising application of NGS is epigenetic profiling. Buenrostro *et al.* have developed a method that uses bacterial transposase for the targeted insertion of sequencing adaptors into areas with open chromatin [44]. This restricted amplification and sequencing allows for the focused profiling of active chromatin regions of the genome. This assay for transposase-accessible chromatin using sequencing (ATAC-seq) could be used to map unbiased chromatin accessibility of specific cell types (antigen-specific T cells, B cells and memory cells) at different timepoints after vaccination and requires minimal cells and minimal hands-on time commitment [45–48]. T cell differentiation is tightly controlled by epigenetic processes. Expression of defined transcription factors (e.g., Tbet, Gata3, RORg, Foxp3, and Bcl6) in developing CD4⁺ T cells confers specific effector functions on the cell (e.g., secretion of Th1-, Th2-, or Th17-specific cytokines) [49]. Cellular immune responses are critical for protection against and/or recovery from intracellular pathogens and virus-specific CD4⁺ T are considered a correlate of protection for herpes zoster vaccine [50]. Genome-wide epigenetic profiling has allowed the development of a better understanding of not only how memory T cell responses are created but also how their qualitative characteristics are imprinted[51]. For example, a recent study found that Fox3P expression was necessary but by itself insufficient for the establishment of Tregs and that a second signal, a specific pattern of CpG hypomethylation, was also required [52]. Other T cell subsets may be under similar regulatory control. One could utilize this information to design a prime-boost vaccination schedule where each formulation optimally provides each necessary signal, resulting in the development of an appropriate, robust, and long-lived population of memory T cells. Epigenetic studies of chronic diseases have identified epigenetic modifications resulting in T cell populations unable to control or eliminate the pathogen [53]. Identifying the problem now enables us to engineer vaccines that can reverse

or counteract this epigenetic programming in order to allow for the development of an effective immune response in patients suffering from chronic infections.

2.3. Simultaneous transcriptome and protein measurement in single cell preparations

Recent advances in NGS have focused on the single cell level (scRNA-seq). This has been transformative for understanding complex cell populations, but scRNA-seq is unable to provide additional phenotypic information such as protein levels of cell surface markers. Additionally, phenotypic cell states may not be observable from scRNA-seq alone due to potential heterogeneity in post-transcriptional and post-translational processes.

Cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) as well as RNA expression and protein sequencing assays (REAP-seq) [54, 55] are new approaches which label antibodies to specific cellular proteins with a combination of 8-bp oligonucleotide barcodes and a poly(dA) sequence, which enables the simultaneous detection of protein and mRNA. Both techniques use droplet microfluidic techniques to simultaneously measure mRNA and protein expression in individual cells.

After cells are stained with DNA-labeled antibodies (Ab), single cells are encapsulated in droplets containing DNA polymerase, oligo-dT, and primers for PCR amplification and sequencing. Cells are lysed upon droplet formation and Ab labels are quantified as part of a transcriptomic readout. One disadvantage for the REAP-seq and CITE-seq approach is that the information regarding the spatial distribution of proteins is lost. A recent commercial method, BD Abseq, applies the Seq-Well approach, but instead of droplets it uses microwells that contain individual cells and barcoded poly(dT) mRNA beads. This arrangement enables efficient cell lysis and reverse transcription of mRNA at the single-cell level [56]. Although there is no practical limit to the number of unique identifiers that can be chemically conjugated to antibodies of choice, these methods are inherently limited by the number of available antibodies, their specificity, the epitope availability, and the number of antibodies that can be introduced per cell before molecular crowding becomes a limiting factor.

Using biological samples comprised of heterogeneous cell types, even in single-subset level and high-throughput single cell NGS, has been an effective approach to deconvolute heterogeneity of cell population because it offers data to identify type, state, and function of cells [57]. However, gene expression is dynamic, and abundance of protein usually has poor correlation with transcript count at the single-cell level [58]. The integration of transcriptomic and proteomic profiling on a single cell can provide a more accurate picture of each cell's immunologic capabilities.

Characterizing the effector functions of antigen-specific cell populations in this manner will allow investigators to directly link transcriptomic activity with cellular function.

2.4. Flow and mass cytometry for immune cell phenotyping

2.4.1. Flow cytometry—The major advantage of flow cytometry has been the ability to assign phenotypic and functional characteristics to single cells. This has been useful in tracking the development, expansion, and acquisition of cellular functions during ongoing

immune responses. Flow cytometry uses antibodies coupled to fluorochromes to detect specific antigens expressed on the cell. Because of emission spectral overlap (i.e., spillover) of fluorochromes in most cases, ~12 colors is an operational limit, although some laboratories have reported 16 or 18. BD Biosciences (San Jose, CA) has made recent advances in the instrument, and dyes claim a 48-color capability (BD FACSymphony); with improved technology, it seems possible that this number could go higher. Based on spectrofluorimetry, spectral flow cytometry (Spectral-FCM) is an emerging technology in flow cytometry (Cytek Aurora). Unlike conventional flow cytometry that detects the peak of emitted fluorescence, spectral-FCM detects the shape of the emitted spectra and analyzes the data with an algorithm instead of the compensation matrices. This new technology can separate emission spectra of fluorochromes with close proximity, such as GFP and FITC, without need for compensation [59]. Cytek Aurora is capable of detecting 48 fluorescent channels by using a three-laser system.

Flow cytometry has been widely used for monitoring immune responses in mixed cell populations and also for tracking phenotypic and functional characteristics of individual T and B cells [60] (including antigen-specific populations). Several optimized multicolor immunofluorescence panels (OMIPs) and comprehensive leukocyte immunophenotyping (CLIP) panels have been developed to obtain phenotypic and functional characterization of hundreds of cell types, including CD4 T helper cell subsets (Th1, Th2, Th9, Th17, Th22, and Tfh), CD8 T cell subsets, regulatory T cell (Treg) subsets, B cell subsets, innate lymphoid cell subsets (ILCs), NK subsets, myeloid/monocytes, and dendritic cell subsets [61]. Tsang *et al.* have used five 15-color CLIP panels (examining 126 cell subpopulations including the Treg, Th17, Th1/2, B cells, monocytes, and dendritic cells) to assess immune responses to pandemic A/H1N1 (pH1N1) vaccine in 63 subjects [62]. These flow-cytometry data were then integrated with the subjects' gene expression, serum cytokine levels, virus titers, and ELISPOT data to develop a predictive model for immune responses to this vaccination [62]. They have demonstrated that an accurate model could be constructed using pre-perturbation cell populations alone, independent of age and preexisting antibody titers [62]. This type of baseline predictive biomarker could be used to identify subjects that will or will not respond to a given vaccine, or even those who are at increased risk of adverse events. The availability of these types of biomarkers could fundamentally change approaches for vaccine administration [62]. Flow cytometry was used to detect the presence of acute plasmablasts (PB) in the blood after immunization with tetanus vaccine [63], inactivated influenza vaccine [64, 65], and yellow fever vaccine [26, 64]. Specific T cells are also recirculating in the blood around the same time period, suggesting that it is a suitable time for capturing antigen-specific lymphocyte populations [64]. This information can inform the development of experimental approaches assessing vaccine immunogenicity by providing detailed phenotypical and functional characterization of the immune cells responding to the vaccine. It can also help elucidate the specific targets of these sorted antigen-specific T cells and plasmablasts. Studying purified cell subsets also enhances the important biological signals by reducing the non-specific activity in mixed cell populations.

2.4.2. Cytometry by time-of-flight (CyTOF)—In mass cytometry (CyTOF), heavy metal isotopes are conjugated to antibodies. These isotopes (typically lanthanides) do not

have the large signal overlap present in many fluorophores because mass spectrometers can readily detect the narrow mass/charge ratios between isotopes [66]. CyTOF approach has the capability to simultaneously detect up to 42 parameters; the development of effective conjugation chemistry for additional metal isotopes could raise that total to 75 [67]. CyTOF starts by labeling fixed cells with antibodies conjugated to metals. Labeled cells are channeled by microfluidics into an inductively coupled mass spectrometer, where the cells are nebulized in argon gas, ionized in a plasma torch, and the resulting ions are passed into a quadrupole filter and a time-of-flight mass spectrometry chamber. The recorded data can then be used to assemble an ion profile for each cell. The presence of heavy metal ions is a marker for the quantity of the protein recognized by the antibody carrying that heavy metal.

This technology has been utilized in the following ways: for phenotypical and functional characterization of pathogen-specific T cells in combination with cytokine expression [68]; for epitope mapping of T cell response in combination with tetramer staining [69]; to evaluate immunity after hepatitis C virus vaccination [70]; to assess TLR signaling pathways and cytokine production after immunization with the split influenza vaccine [71]; and to evaluate contribution of non-heritable versus heritable factors [72]. Brodin *et al.* have included the response to influenza vaccination among many other variables [72].

2.5. Luminex and Mesoscale for cytokine determination

Cytokines, small proteins released by immune cells, have an important role in cell signaling and intercellular communication, as well as in controlling cell growth, activation, migration, and differentiation. Chemokines are a type of cytokines that induce chemotaxis (i.e., influences the migration of cells). Messages in the immune system are transported by cytokines and chemokines. There are many technologies that make multiplex cytokine detection and the related analysis possible. Luminex multi-analyte profiling (xMap; Thermo Fisher Scientific: Waltham, MA) and the Mesoscale Discovery (MSD; Mesoscale Diagnostics: Rockville, MD) platforms can detect tens or hundreds of analytes. xMAP technology is a bead-based assay using different sets of microspheres in a liquid suspension [73]. Microsphere sets are internally dyed with two to three spectrally different fluorophores, producing up to 500-member microsphere sets [74]. Each set of microspheres has a unique spectral signature that is determined by concentration of their internal dyes, which determine analyte specificity. The surface of each microsphere set can be coated with different reagents specific for various bioassays such as enzyme assays and nucleic acid assays. Similar to a multiplexed ELISA, the MSD platform captures antibodies for the analytes of interest, which are then coated on a microtiter plate. In this platform, cytokines are detected by electrochemiluminescent tags on analyte-specific antibodies. This method has several advantages: high sensitivity, low background, minimal washing steps, and simultaneous, multi-analyte detection capability [75]. These technologies have been used in the following ways: to study the responses to purified protein derivative (PPD) in Bacille Calmette-Guerin (BCG)-vaccinated infants and unvaccinated controls [76]; to characterize CD4 T helper cell responses to the yellow fever vaccine [77]; to investigate the levels of cytokines and chemokines after influenza vaccination [78]; and to compare differences in cytokine response and biosignatures of Malawian infants and infants in the UK following BCG vaccination, which may indicate variability in the protection conferred by infant BCG

vaccination [79]. Luminex technology has been used to characterize differences in cytokine responses to standard childhood vaccines, including tetanus, diphtheria, acellular pertussis (DTaP), 7-valent pneumococcal conjugate (PCV7) vaccines, and hepatitis B vaccine [80]. These studies, combined with genotyping of cytokine and cytokine receptor gene polymorphisms have found that vaccine-induced immune responses in infants (and potentially adults) may be influenced by these genetic polymorphisms. This type of work exemplifies the hypothesis-generating nature of some systems biology work. With new information, it will now be possible to explore the functional effects of the identified SNPs. Mesoscale Discovery was used for cytokine profiling of the rVSV-Zaire Ebolavirus (ZEBOV) vaccine candidate [81]. *In vitro* stimulation of PBMC samples from vaccinated human subjects identified a cytokine signature that, in combination with circulating follicular helper T cell signature, may correlate with protection developed by rVSV-ZEBOV vaccine [81]. The development of a T cell signature correlated with protection suggests that the inclusion of T helper epitopes will enhance vaccine efficacy, and should be considered in the design of next-generation vaccines against filoviruses.

Mesoscale and Luminex assays, like many of the high-throughput assays described in this review, require limited sample volumes, which is a significant advantage when conducting studies where only limited amounts of cells or serum are available (i.e., pediatric populations).

2.6. Protein and peptide microarrays for antibody profiling

The monitoring of antibodies and the antibody repertoire following vaccination is a powerful method to assess humoral immunity and identify diagnostic markers and “go/no go” decision points for vaccine candidates. Antibody profiles can help determine an individual’s past exposure history.

Microarray assay refers to the miniaturization of thousands of assays on a small plate. It is most often used in the context of gene expression microarrays (with tens of thousands of oligonucleotide probes on a slide) but also applies to antibody microarrays (where antibodies are immobilized on plate and used to detect antigens). In peptide and protein microarrays, libraries of peptides or proteins are immobilized on slides or plates to detect the presence of specific antibodies or ligands that bind to the immobilized molecules [82]. Proteins are more fragile and delicate when compared to peptides, and fabricating a protein library is much more complex and time-consuming. The ease of development and affordability of peptide arrays has resulted in their being the more popular and effective tool in proteomics studies [83].

2.6.1. Peptide microarrays—Peptide arrays have been utilized to profile antibody responses in vaccinated individuals, to develop new vaccines, and to monitor clinical interventions. Peptide arrays were used in HIV-1 vaccinated and infected patients [84] to successfully study antibody diversity, suggesting that similar assays may be useful in preclinical and clinical HIV-1 vaccine studies. In one study of seasonal influenza vaccine, peptide arrays were successfully used to identify pre-existing antibodies to the vaccine and to differentiate responders/non-responders based on patterns and intensity of response [85,

86]. Furman *et al.*, in their systems biology approach, identified specific variables associated with serological vaccine response that, with 84% accuracy, predict antibody response [85]. Price *et al.* identified peptide epitopes that correlated inversely with age and seasonal A/H1N1 neutralization titer, indicating pre-existing immunity to selected peptides, along with age, can be used as a predictive biomarker of vaccine response [86]. A peptide microarray was also used to identify immunodominant B cell epitopes within the pertactin of *Bordetella pertussis* induced by cellular (miPc) or acellular (miPa) immunization [87]. It is believed that antigenic variation in *Bordetella* isolates has reduced vaccine effectiveness and is a contributor to the increased number of cases. Importantly, reported antigenic differences between vaccine-based and circulating strains of *Bordetella pertussis* indicate that mutations in pertactin do not affect the immune responses elicited by either the Pc or the Pa vaccines, suggesting that absence of these neutralizing Ab epitopes in some *Bordetella* strains is not a contributing factor in decreased vaccine efficacy.

2.6.2. Protein Microarray—Protein microarray has been used in the following ways: to discover schistosomiasis vaccine antigens in Brazilian patients with chronic infection [88]; to identify immunogenic proteins of *Plasmodium vivax* [89]; to detect immunodominant antigens in the membrane of the *Salmonella typhi* in typhoid fever patients [90]; and to recognize strain-specific B-cell epitopes of *Trypanozoma cruzi* in patients with Chagas disease [91]. These studies highlight the ability of protein/peptide microarrays to facilitate the identification of new, promising antigens for vaccine development. Similar studies profiling humoral responses to the smallpox vaccine clearly demonstrated that humoral immunity largely targeted about two dozen of the nearly 250 proteins encoded for by the vaccinia genome [92]. This allows for the creation of diagnostic biomarkers of response or protein/peptide-based vaccines that circumvent the lengthy list of contraindications for this live virus vaccine [92].

2.7. Metabolomics for immune cell metabolic state

Metabolomics is the identification and profiling of small compound metabolites within biological samples. Metabolites in a metabolome create a metabolic-reaction network in which outputs of one chemical reaction may be inputs for another chemical reaction. Therefore, the metabolome is time sensitive and much more dynamic than the proteome and genome. To better understand an organism's metabolic activity, it's critical to study the smaller molecules that are influenced by the genome, proteome, microbiota, and environmental factors. Metabolomics have been investigated by two approaches: targeted metabolomics, which quantifies defined sets of known and annotated metabolites by using current knowledge of biochemical pathways; and untargeted metabolomics, which analyzes a sample's known and unknown measurable analytes [93]. The targeted metabolomics technology needs calibration with highly purified, stable, and well-defined standards [94]. Untargeted metabolomics does not measure quantity of specific compounds but does detect and provide relative abundances of up to thousands of compounds. The analysis yields of untargeted metabolomics have demonstrated very reproducible results even by using different protocols and experimental approaches [95].

Both nuclear magnetic resonance (NMR) and mass spectrometry (MS) are used to generate metabolomic data [96]. Both techniques provide the spectra of compounds that exist in the analyzed sample.

Metabolomic profiling of hepatitis C-infected individuals can distinguish between patients with pneumonia [97] from those who have sepsis [98]. An accurate diagnosis is essential for proper medical treatment, and similar diagnostic tests can be developed to quickly assess immune responses to vaccines. Metabolic studies of vaccinia virus (VACV) replication showed that the inhibition of glutamine metabolism blocked VACV protein synthesis, a finding that may lead to novel therapeutic options for poxvirus infection [99]. Metabolomic studies have also highlighted molecular signatures of vaccine adjuvants [100], information that can be used to appropriately combine adjuvants with vaccine components to shape immune responses in desired directions. Metabolomic studies have also been used to identify biomarkers of adverse reactions following vaccination [101]. This suggests that alterations in metabolic activity might be an approach to minimize adverse events to vaccines, especially in at-risk individuals. Although this technology has not been applied as widely as other -omics technologies, metabolomics has great potential in vaccine development and can provide insights into the mechanisms underlying vaccine-induced responses [102]. Metabolic changes could be related to vaccine efficacy, safety, and plausible adverse effects of vaccine [102]. Response to immunization has been monitored by metabolomics [103]. Cui *et al.* reported that dengue virus patients have alteration levels of serum metabolites and that treatment of disease results in a return to normal levels [103]. These results suggest that metabolite levels may serve as a prognostic indicator of disease severity. Recently, Li *et al.* used a systems biology approach in live attenuated Herpes zoster vaccine (Zostavax) to show significant alterations in several metabolic pathways, with more significant changes on Days 1 and 14 after vaccination [30]. Given the metabolic burden that activation, differentiation, and clonal expansion of antigen-specific T and B cells triggers, it is not surprising that metabolism and metabolic activity play a role in shaping immune responses. Li *et al.* quantified that effect and highlighted the involvement of specific components that can be investigated as potential biomarkers [30].

3. Analysis of complex data (Handling of data and creating predictive models)

Methods from network theory have the ability to improve predictive models of immune response by identifying important genes or gene sets based on their connectivity with other genes. A biological network is a set of nodes and edges connecting them. The nodes often represent gene products, but they may also be cell types, nucleotide variants, epigenetic markers, metabolites, microbiota, brain regions, and a variety of other types depending on available data. The edges in a biological network define how the nodes are connected, which also may be defined in numerous ways depending on the data. While one focus is on the genes as nodes in expression data, the edges in these gene networks may be defined in a variety of ways, such as gene regulation (directed edges), gene-gene interaction, or correlation (undirected edges). In the most widely used gene network, the co-expression

network, edge weights between genes are determined by the correlation across subjects in a gene-expression study.

The centrality of a gene in a co-expression network is an indication of its influence in the network (e.g., the number of edges), and hub genes may be important predictors of disease status [104] or other phenotypes [105] including vaccine immune response. Because genes that share a common function are more likely to be co-expressed [106], clustering is an effective method to identify important functional genesets or modules. There are numerous methods for clustering, such as tree-based [107] and modularity-based [108], but the common goal of these methods is to find groups of genes that are more closely connected to each other than to genes outside the cluster. The variation of genes within these clusters may then be collapsed into a single unit of analysis by geneset variation methods, like single-subject geneset enrichment analysis (ssGSEA) [109]. These collapsed genesets can then be used as predictors of disease phenotypes [110] or immune response [111].

A related type of biological connectivity is differential co-expression [112], which incorporates information about the phenotype into the connection strength. Networks can be constructed from these interactions using gene expression [113] or genome-wide association study (GWAS) data, and the hubs from these networks may be important immune response regulators that provide additional information beyond co-expression by including immune response phenotypes [114]. In addition, these network approaches can incorporate prior knowledge based on functional interaction information [115, 116]. Interactions in gene expression and GWAS may be integrated in eQTL to further characterize regulatory effects of interactions [117] or effects of vaccine immune response [118]. Like differential expression, it should be noted that co-expression and interaction effects can be influenced by biological effects not related to the phenotype, such as age or cell-type heterogeneity.

It is rare for a single gene (or protein or metabolite) alone to drive the expression of a phenotypic effect, which is one of the advantages of a geneset approach. Machine learning methods can incorporate genesets to predict immune response, or they may learn complex models directly from the individual genes. Two common machine learning methods are the tree-based random forest [119] and the regression model-based Lasso (least absolute shrinkage and selection operator) [120]. Lasso performs feature selection during model fitting through the shrinkage of regression parameters, but it is parametric and generally assumes that features are independent, which may restrict its exploration of more complex models. Random forest has fewer model constraints than regression. While this is useful in cases where multivariate genetic architecture is involved in the phenotype, it is less interpretable than Lasso. Random forest can also integrate heterogeneous variable types [121], which is beneficial for the analysis of multi-assay data. The objective of machine learning methods is to obtain a model that predicts with minimum error; however, machine learning models are prone to overfitting. Thus, cross-validation is utilized to provide a realistic assessment of the quality of a machine learning model. Models can be further improved by feature selection but should be wrapped into nested cross-validation [122] or in a differential privacy approach [123] to provide realistic accuracy estimates.

4. Integrating systems biology approaches into clinical trials

Systems biology approaches complement reductionist approaches by illustrating the interactions between processes and components of the immune system critical to the development of protective immunity after vaccination [124]. The inclusion of systems biology (i.e., bioinformatics and computational modeling) as a principal part of the clinical-trial process may provide immense advantages for vaccine development. These studies, for example, might suggest a more comprehensive assessment of humoral immunity (avidity, Ig repertoire, plasmablast ELISPOT response) in addition to measuring a serum IgG titer. Similarly, they might suggest a specific target population that should be studied or highlight inclusion/exclusion criteria that should be included in the protocol. Systems biology studies may also inform the appropriate timing of interventions, study visits, and biological sampling.

By applying the knowledge gained through systems biology approaches, clinical trials can be designed based on a predictive understanding of the correlates, mechanisms, and molecular signatures of vaccine-induced protective immunity. For example, the expression levels of genes identified in the yellow fever vaccine (YF-17D) study had very high predictive power for antibody and CD8⁺ T cell responses and may serve as useful early stage biomarkers of vaccine response and/or efficacy [26]. Another example is the identification of an innate transcriptomic signature involving TLR7 and elements of the TLR7/8 signaling pathway [125], predictive of increased influenza vaccine-induced HAI titer, which may support the usage of TLR 7/8 agonist-adjuvanted (imiquimod and/or VTX-2337) influenza vaccines to elicit protective immunity in poor responder populations such as the elderly. Utilizing TLR ligands (TLR4 and TLR7) as influenza vaccine adjuvants induces robust protective Th1- and Th2-type responses against the viral hemagglutinin in mice [126]. Recent reports describe adjuvanted trivalent influenza vaccines that induced high-titer serum antibodies in human trials [127–129]. The results from a double-blind, randomized controlled trial demonstrated that the use of TLR agonists does enhance humoral responses to influenza vaccines, providing direct confirmation that systems biology-derived insights can be used to improve influenza vaccines [129]. The ability to predict and understand inter-individual variations in immune responses to vaccines may allow us to reverse engineer novel vaccine and therapeutic candidates and to define correlates of protection in interventional clinical trials [130–132]. For many vaccines (e.g., influenza, MMR, hepatitis, rabies, polio, and others), antibody titers serve as well-defined correlates of protection; however, for some pathogens (such as malaria, tuberculosis, norovirus, HIV), there are still no established correlates of immunity/protection and antibody measurements may not be the only relevant correlate of protection [131, 133]. Innovative terms for immune correlates of protection following vaccination have been recently proposed by Plotkin and Gilbert (i.e., mechanistic, which is causally responsible for protection; and non-mechanistic, which is not mechanistically responsible for protection) [50]. Examples of non-mechanistic correlates of protection include antibody response (measured by ELISA) to meningococcal vaccine, antibody response (measured by VZV-gpELISA) to varicella zoster virus vaccine, and serum IgA response to rotavirus vaccine [50]. Hence, the incorporation of systems biology approaches into clinical trials may be useful for identification of early predictors of vaccine

efficacy and defining novel correlates of protection. For example, the proof-of-concept for reverse engineering and structural-based vaccine antigen-design approaches have been applied to develop a candidate respiratory syncytial virus fusion F glycoprotein (RSV F) subunit vaccine eliciting neutralizing antibodies associated with protection from respiratory disease [134]. We and others have published examples on how systems biology/vaccinology can be integrated into clinical trials, identify correlates of protection, and inform vaccine development for worldwide disease prevention [131, 132].

Given the high cost of omics assays, systems biology-based clinical trials may steer away from testing a small number of outcomes on thousands of individuals. Instead, there may be iterative studies on smaller cohorts of subjects with large numbers of immune outcomes. Each subsequent trial would be informed by the data and analysis that come from the preceding trial (Figure 2).

5. Challenges

The new technologies and approaches described in this review have the potential to revolutionize the way that new vaccines are designed, developed, tested, and used. Recent work involving nucleic acid vaccines highlights one example of how these methods can be implemented. The development of DNA vaccines has been hampered by poor immunogenicity, the need for large antigen doses, and/or boosting with protein-based vaccines. One approach that has been shown to induce stronger immune responses to West Nile virus (WNV) and YFV is the use of infectious DNA (iDNA), which consists of a plasmid containing a complete viral genome under the control of transcription elements that allow the establishment of a productive infection in susceptible host cells [135]. Consensus sequences can often be used in plasmid construction such that immune responses will be cross-reactive against multiple pathogen strains. This has been successfully done with the GLS-5700 Zika DNA vaccine that is in clinical trials [136]. Similar advances have been applied to the development of an intradermally delivered Zika mRNA vaccine containing lipid nanoparticles [137]. This vaccine takes advantage of genomic information to optimize codon usage. This approach increases mRNA stability and decreases detection and elimination by intracellular innate immune responses, thereby enhancing and prolonging antigen production [138]. A single dose of this vaccine elicits high titer neutralizing antibody responses and provides protective immunity in non-human primates. Another Zika virus vaccine contains self-amplifying mRNA based on alphavirus amplicons [139]. The mRNA construct produces significant quantities of antigen and also triggers a strong innate immune response, acting as its own adjuvant.

Further understanding of adjuvants and innate immune function may lead to the creation adjuvanted DNA vaccines that elicit high affinity IgG responses as well as robust cellular immunity [140]. A newly licensed zoster vaccine contains the AS01 adjuvant system comprised of liposomes, MPL, and QS-21 [141]. This adjuvant system is known to boost T cell responses, which are the putative correlate of protection from zoster. Clinical testing has revealed that this vaccine demonstrates superior immunogenicity, seroconversion, and efficacy in older adults. Knowledge about pathogen biology, host response, and adjuvant activity allowed researchers to select the viral protein (glycoprotein E) targeted by both

neutralizing Ab and T cell response, determine a presumed correlate of protection (CD4+ T cell responses and not neutralizing antibody), and include an adjuvant known to enhance T cell activity. The final result is an incredibly effective subunit vaccine with an improved efficacy profile over the existing vaccine, especially in older adults. Another challenge in the field is the rapidly growing scope of the datasets created by the omics technologies. Our ability to handle, evaluate, and understand complex data is lagging behind our ability to create the data, and gold-standard analytical approaches have yet to be developed. This is an area of active investigation; many of the statistical, bioinformatics, and computational approaches described above provide an excellent starting point for mining and interpreting increasingly high dimensional data. As the methodologies discussed in this review are increasingly applied to complex problems in vaccinology, the field is likely to see similar success stories.

6. Conclusion

The empirical approach of vaccine design (i.e., administration of killed or attenuated microbe or toxins) has been used to develop many current vaccines and has save millions of lives; however, this approach does not address several emerging infectious diseases and pathogens with complex biology. The human immune system is complex, and it remains challenging to predict specific details of the immune response to a given vaccine or pathogen. New discoveries in immunology and microbiology may lead to better understanding of immune response to pathogens and vaccines. This knowledge, along with the use of new technologies, has led to the emergence of new adjuvants and synthetic peptides, as well as glycoconjugate vaccines and DNA vaccines. Despite these advances, vaccines against complex diseases such as HIV, malaria, tuberculosis, and dengue have failed—usually with no understanding of reasons for failure. To fill this knowledge gap and have a deeper understanding of mechanisms that govern immune response to vaccines, vaccinologists turn to systems biology approaches.

The main goal of a systems biology approach in vaccine development is to integrate data from different biological sources and extract meaningful information useful in rationally designing new vaccines, as well as predicting immune responses. This approach collects data from new, high-throughput “omics” methods to gather unbiased information from baseline and post perturbation (vaccination) states of the immune system. Then, by using statistical methods and computational modeling, investigators try to identify correlations between the measured elements and discover previously unrecognized functions and effects. For example, expression profiling has been used to understand the unique effects of different adjuvants by identifying which biological pathways are activated by compounds such as alum, CpG, or MF59 [142]. This information allows investigators to pair appropriate adjuvants with vaccines in order to influence the resulting immune response toward protective effects. Another important contribution of systems biology has been a better understanding of the connections between metabolism, age, microbiome, concurrent infections, and immune response to vaccines [131]. This information will assist in the development of vaccines targeted to specific population subgroups, much like what is already happening with influenza vaccines (e.g., vaccines geared for adults over 65 years of age or for people who have egg allergies). Systems biology studies have led to the discovery

of biomarkers or signatures of immune status and capability. These signatures have been shown to correlate with the outcomes of vaccination and can be used in future clinical trials as predictors of efficacy. Systems biology has also broadened scientists' view of appropriate immune outcomes to test. Instead of simply measuring total serum IgG, investigators are now evaluating antibody responses in terms of neutralization, complement fixation, antibody dependent cellular cytotoxicity, and antibody dependent cellular phagocytosis, while also studying the specific characteristics of Ab molecules that contribute to these myriad functions (e.g., isotype, affinity, subclass, glycosylation) [143, 144]. This information is critical given the lack correlates of protection against many pathogens. Similar expanded capabilities are being created in the study of cellular immunity. Collectively, these efforts will enable the scientific community to identify and establish correlates of protection for new (and existing) vaccines, greatly facilitating vaccine evaluation. As outlined in this review, systems biology approaches to vaccine development ("systems vaccinology") is a young field. While a vaccine has not yet been created using systems biology approaches, the understanding of the complex sequence of events required for protective immunity has greatly increased, and it is this information that will be essential for the rational design of safe and effective vaccines. These technologies and computational tools are experiencing rapid advances in capability, accessibility (particularly in lower costs), and use among investigators; therefore, one can expect this field to yield exciting discoveries as it moves forward.

7. Expert Commentary

Vaccinologists are struggling to develop vaccines against many of the world's pathogens that threaten human health, such as hyper-variable viruses, malaria, TB, and many others. Systems biology represents a holistic, newer tool in the armamentarium of vaccinologists. As illustrated in this review, this tool has already proven of value by elucidating details regarding how immune responses are generated and by what mechanisms, as well as by providing molecular/genetic signatures of normal and aberrant immune responses. Such knowledge can be leveraged to reverse engineer novel vaccine candidates for testing; however, major challenges remain regarding the full use of this tool in vaccine development. These include more sophisticated annotation of gene and other molecular entities for use in designing vaccines. In addition, the field is in desperate need of standardized, validated, less expensive, and useful bioinformatic approaches to mining the considerable amounts of data generated by systems-level experiments. A significant limitation in these experiments is adequate funding to conduct valid experiments. Whereas the past experimental paradigm was to conduct a few assays on hundreds to thousands of subjects, this new paradigm requires thousands of assays on tens of subjects. High-throughput omics technologies can cost thousands of dollars per assay or experiment, which limits the number, validity, and generalizability of the experimental findings.

Another important insight is that systems vaccinology will further fuel the need for a scientific team approach to study design and interpretation. In our own studies, this work has required clinicians, immunologists, statistical genetics experts, bioinformaticians, and others. Thus, while the informational content of systems biology is great, so are the costs involved in generating, interpreting, and communicating the resultant data.

Finally, the results of these studies are highly likely to further advance the knowledge toward individualized vaccinology. The idea is that by studying groups of individuals with systems approaches, we will begin to understand (and predict) specific phenotypes that identify differential immune responses, and therefore inform vaccine practice.

8. Five-year view

We believe that systems biology approaches will facilitate a deeper understanding of—and ability to interpret—vaccine-induced immune responses, and these responses will continue to evolve in usefulness. Increasingly sensitive assays (including omics assays and single-cell genomics) will lead to a vastly more sophisticated understanding of the generation and maintenance of protective, non-protective, and even aberrant immune responses. Thanks to the development of sophisticated bioinformatic approaches to interpreting systems-level data, it's possible to mine the data for insights not currently obtainable. Taken together, these advances will enable the identification of molecular signatures that predict the likelihood of vaccine response to an antigen, number of vaccine doses required, the likelihood of an aberrant immune response, and other factors. Such data will also allow investigators to understand molecular/genetic restrictions to developing and maintaining protective immune responses and reverse-engineer novel vaccine candidates.

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Key issues

- Systems biology is being applied to studies of vaccine-induced immune responses.
- Such studies have led to novel insights into how immunity is generated to viral vaccines.
- Newer assays are being incorporated into systems-level studies. This includes single-cell techniques, RNA- and DNA-based assays, epigenetics, and massive cytokine/chemokine and flow cytometry measures, as well as others.
- Bioinformatic approaches that are reproducible, standardized, and validated are needed to better interpret the results of systems-level studies.
- The end goal of systems vaccinology is deep insight into how vaccine-induced immunity is generated, and identification of barriers to developing such immunity; in turn, this will allow reverse-engineering of novel vaccine candidates.

Vaccine design: Empirical approach

Figure 1. Comparison of classical and system biology approaches for vaccine development. The classical approach to vaccine development involved the isolation, inactivation (or attenuation or killing) of pathogen, and its injection into the host. Trial and error led to a vaccine formulation that provided protection against that pathogen but was not typically accompanied by an understanding of biological mechanisms underlying that protection. This lack of understanding often led to failures or side effects of the vaccines. In a systems biology approach, researchers use modern high-throughput technologies to collect rich datasets characterizing epigenetic, transcriptomic, proteomic, and metabolomics events that occur during an immune response to pathogens, microbiota, and vaccines. These data provide insights not only into the cell subsets and/or tissues involved, but also the complex interactions required for the development of immunity. The analysis, interrogation, and integration of these data allow investigators to elucidate and understand the biological rules that govern immune responses to pathogen and/or vaccines. The integrated data can be used

to create predictive models of immune responses. These models can then be utilized in the directed development of a new generation of vaccines likely to elicit critical immune pathways leading to protective immunity. The resulting formulations are then used to immunize animal models and eventually humans. Data from these animal studies and clinical trials are then used to refine the predictive models and “rules” governing the immune system in an iterative cycle. (Adapted from Oberg AL, Kennedy RB, Ovsyannikova IG, Poland GA. Systems biology approaches to new vaccine development. *Curr Opin Immunol*. 2011 Jun;23(3):436–43. doi: [10.1016/j.coi.2011.04.005](https://doi.org/10.1016/j.coi.2011.04.005).)

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Figure 2. Overview of systems biology approach in development of new vaccines.

Biological specimens are collected from a study cohort before and after vaccination in order to evaluate the vaccine's effect on the immune system. The timing and nature of the specimens is determined by known biology, the nature of the study, and the intent of the investigators. High-throughput technologies are employed to profile different components of the immune system in response to vaccination. The high-throughput data is then analyzed and integrated by bioinformatic tools to identify new biomarkers and molecular signatures. These data are also used to create predictive models of immune response and may also identify more relevant study endpoints. The insights gained from integrated data and results from the predictive models are then used to design and develop new vaccines with predicted immunogenicity and safety, which can then be validated in pre-clinical experiments, animal models, and clinical trials. These studies then serve as the starting point for the next round of scientific discovery. This iterative process builds a detailed understanding of how the individual immune system components react and interact during ongoing immune responses. This knowledge is used to improve the predictive models and design the next set of studies, thereby providing a directed approach to vaccine development and testing. Clinical trials ensure the immunogenicity, safety, and also longevity of immune response of new vaccines before its public administration.