Systems vaccinology and big data in the vaccine development chain

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Summary

Systems vaccinology has proven a fascinating development in the last decade. Where traditionally vaccine development has been dominated by trial and error, systems vaccinology is a tool that provides novel and comprehensive understanding if properly used. Data sets retrieved from systems-based studies endorse rational design and effective development of safe and efficacious vaccines. In this review we first describe different omics-techniques that form the pillars of systems vaccinology. In the second part, the application of systems vaccinology in the different stages of vaccine development is described. Overall, this review shows that systems vaccinology has become an important tool anywhere in the vaccine development chain.

Keywords: bioinformatics; proteomics; T-cell; transcriptomics; vaccination.

Introduction

There are many effective vaccines available to protect humans against infectious diseases. However, the development of vaccines against 'difficult' pathogens remains challenging. For example, vaccines against human immunodeficiency virus (HIV) and respiratory syncytial virus (RSV) are still under development. Furthermore, some existing vaccines require improved efficacy, e.g. vaccines against *Bordetella pertussis*, influenza viruses, *Mycobacterium tuberculosis* and *Plasmodium* species causing malaria. In addition, new infectious diseases such as severe acute respiratory syndrome and Ebola virus disease will continue to emerge, which require new vaccines to prevent epidemics. Also, therapeutic vaccines against non-infectious diseases, such as cancer, are needed. The lack of in-depth knowledge of the pathogen and requirements for protective immunity often hamper development.[1] Application of systems biology during the development of vaccines, or systems vaccinology, can be an important tool to enhance insight into immune responses induced by (candidate) vaccines or identification of (early) correlates of protection.[2,3] Alan Aderem defined systems biology as a 'comprehensive quantitative analysis of the manner in which all the components of a biological system interact functionally over time and space that is executed by an interdisciplinary team of investigators.'[4] This definition can readily be applied to study the responses to

vaccination. Systems-based approaches are often labeled unbiased and broad (sometimes even the word 'holistic' is used). However, that is only true to a certain extent, as defining a research objective is already introducing bias. Often only a few analytical techniques are used (as described in the next section) to address the objectives (Fig. 1). Usually, there are also limitations in availability of materials. For example, in preclinical studies using mice the amount of blood that can be collected is very limited, whereas in clinical studies mainly body fluids can be used, although biopsies and mucosal lavages are sometimes available. Also, time is an essential parameter of systems vaccinology for studying kinetics and cause–effect relations, but repeated sampling is limited in humans. In this review, we describe current developments of the techniques that form the pillars of systems vaccinology and discuss the implementation of systems vaccinology in the vaccine research and development chain.

Systems vaccinology techniques

Twenty years ago, Rino Rappuoli and co-workers introduced reverse vaccinology, a genome-based method to identify new leads with strong antibody responses.[5] However, the role of immunogens is currently studied in conjunction with other factors, such as adjuvants and antigen delivery, as these influence immune responses and induction of immunological memory that are key for vaccine
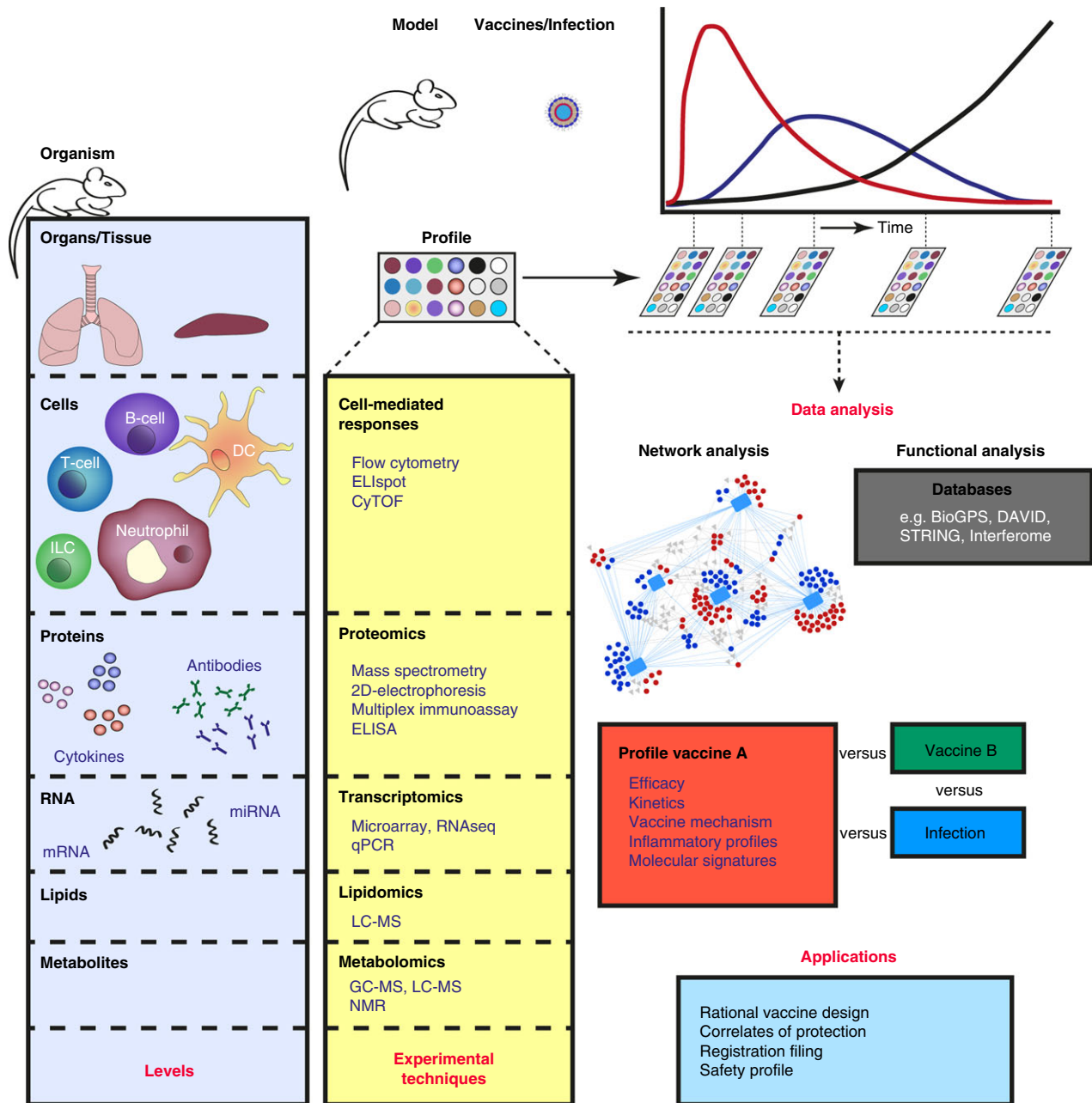
Figure 1. Systems vaccinology approach in a pre-clinical setting. A biological system can range from a single cell to the complete human body consisting of different levels such as genes, proteins, cells, tissues and organs that interact with each other. The biological processes in these levels have distinct time- and space-resolved kinetics. Information on the immune status can be acquired by analysis at the molecular level of the actors (i.e. gene expression, protein synthesis, lipid secretion and production of metabolites), or by determining the changes in cellular composition and morphology. To study the relationship and interaction between all distinct levels of a biological system, a comprehensive approach is required, using multiple analytical techniques. Data, preferably obtained during a time course of the same subject, are combined for further analysis. Network analysis (e.g. Cytoscape) is performed to determine co-expression profiles, indicating interdependence. Functional analysis is executed in public databases, e.g. DAVID (www.david.ncifcrf.gov), STRING (www.string-db.org), BioGPS (www.biogps.org), and Interferome (www.interferome.org). Combined data form a response profile for a vaccine. Vaccine profiles can be compared with other vaccine or infection profiles and used for multiple applications as mentioned in Table 1.

efficacy. Systems vaccinology addresses these aspects in order to understand why some vaccines function properly whereas other vaccines do not. In this section, the most important and widely used techniques applied in different levels of systems vaccinology are described. An overview of these techniques and applications thereof that are used for the systems vaccinology-based approach, are listed in Table 1.

Table 1. The omics in vaccinology

Omics type	Application	Technology	Literature
Genomics			142
	Single-nucleotide polymorphism	Restriction fragment length polymorphism (RFLP)	143
		Microarray analysis	144, 145
		DNA sequencing	
Epigenomics	Age-related immune responses, vaccine-induced memory T-cells	ChIP-seq, DNA methylation	146, 147, 148
Personal genomics	Personalized vaccines		149, 150, 151
Transcriptomics			152, 153
	Single cell transcriptomics	RNA-seq	154
		CITE-seq	17
	Host–pathogen interaction	RNA-seq, Microarray	155, 156
	Infection-induced responses	RNA-seq, Microarray, quantitative polymerase chain reaction (qPCR)	12, 157
	Transcriptional responses by vaccination	RNA-seq, Microarray, qPCR	13, 15, 116, 124, 126
	In vivo analysis of transcriptome	Transcriptome *in vivo* analysis (TIVA)	11
Proteomics			
Immunoproteomics	T-cell epitope identification	Liquid chromatography-mass spectrometry (LC-MS)	20
	Antibody specificity (antigen identification)	ELISA, MIA, two-dimensional Western blotting + LC-MS	59
Interactomics and Ligandomics	B-cell epitope mapping	X-ray crystallography	158
		LC-MS (epitope excision/extraction)	159, 160
	Antibody epitope identification	Peptide arrays	60
	Antigen identification	Immunoglobulin sequencing	55
	Cell-to-cell signaling	Chemical cross-linking mass spectrometry (XL-MS)	36
Peptidomics	MHC epitope display	Mass spectrometry	161
Secretomics	Chemokine secretion	BONCAT	40
Systems serology	Antibody level, isotype, subtype, specificity, functionality, glycosylation	ELISA, MIA, LC-MS, Western blotting	72
Metabolomics			47
Metabolomics	Response to vaccination Predictive biomarker discovery	Gas chromatography-Mass spectrometry (GC-MS) LC-MS Nuclear magnetic resonance (NMR)	46, 162, 163
Lipidomics	Biomarker discovery	LC-MS	105, 164, 165
Glycomics	Mapping antibody glycosylation	Capillary electrophoresis LC-MS	166
Cellomics	Mechanism of action, Correlates of protection, Involved cell types	Flow cytometry, ELIspot Cytometry by time-of-flight MS	12, 15 53, 54, 137
	B-cell and T-cell repertoire	High-throughput single-cell analysis	56, 57, 58
Microbiomics	Vaccine optimization Environmental effects Host-interaction	Next-generation sequencing	167, 168, 169, 170, 171, 172, 173 and 174

Transcriptomics

For decades, RNA analysis has been applied to study vaccine-induced responses[6] and has evolved from (quantitative) polymerase chain reaction to high-throughput methods like microarrays and next-generation sequencing (RNAseq). The applications range from assessing transcriptional profiles in whole tissues and blood to

purified cell populations[7,8] and, more recently, even single cells.[9,10] In contrast to conventional transcriptome analysis that results in loss of tissue or cells, transcriptome *in vivo* analysis allows us to monitor mRNA expression *in situ* in live cells or animals.[11] Transcriptomics of the complete genome is unbiased, relatively straightforward and is offered as a service by many contract laboratories. Gene expression levels and kinetics provide information on involvement of genes following immunization.[12–15] However, mRNA is often not an effector molecule in itself, but needs translation into protein and mRNA levels often do not correlate with protein content. One study reported that in mice, under those conditions, only 27% of the mRNA transcript correlates with protein expression.[16] To that end, transcriptomic profiling in vaccine research serves as an excellent tool to find potential markers of vaccine-induced responses that subsequently need to be confirmed on protein or cellular level. Novel methods such as cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) try to overcome this translational challenge by combining in parallel the targeting of single cells with protein markers with unbiased transcriptome profiling to obtain more specific information on gene expression in cellular phenotypes than RNA-seq in the complete tissue.[17] In addition to mRNA profiles, investigating the role of non-coding microRNAs becomes relevant given their role in immunology.[18]

Proteomics

Contrary to the transcriptome, the proteome encompasses the effectors that exert and control the immune response. Receptors, ligands, enzymes, hormones and even structural proteins detect foreign substances, determine the identity of cells and communicate messages between cells and tissues. Profiling the subset of proteins and peptides involved in the immune response (termed immunoproteomics)[19] is important to understand how vaccines work. During the last three decades, mass spectrometry (MS) -based proteomics has emerged as an essential tool to profile this immunoproteome in terms of protein identification, protein dynamics and protein–protein interactions. Hunt *et al.*[20] pioneered the MS-based analysis of immunogenic peptides presented by molecules of the major histocompatibility complex (MHC). Since then, many studies have profiled the human leucocyte antigen (HLA) ligand repertoire using immunoprecipitation techniques and subsequent MS-based ligand identification to find new antigen leads and to understand the concept of immunogenicity. Apart from targeting infectious diseases,[21–24] MHC ligandome analysis also is applied in, for example, cancer immunotherapy.[25] Recent studies, however, have shown that the plethora of MHC-presented peptides is even more complex than expected, due to the formation of non-linear peptide sequences (i.e. splicing

variants) of these T-cell epitopes as it is estimated that one-third of the CD8^{+} T-cell epitopes is comprised of proteasome-generated spliced epitopes.[26–29]

In addition to profiling the T-cell epitope repertoire, assessing the antigenic determinants that interact with B-cells and antibodies is of high significance in elucidating immune responses in vaccine development and vaccine design.[30–32] Since B-cell epitopes are often conformational or even discontinuous, their identification is complex and currently not possible using *in silico* tools. With instrumental improvement of MS and the supporting software tools, MS is now used as a versatile tool to determine B-cell epitopes, that is, the areas where antibodies bind. Opuni *et al.*[33] comprehensively reviewed MS-based approaches to map B-cell epitopes. Antibody–antigen binding is just one example of a protein–protein interaction. Large-scale interaction analysis (interactomics) is rapidly developing to identify any type of protein–protein interaction.

A new class of MS-cleavable cross-linking reagents has a great potential in interactomics.[34–36] Like all other cross-linking reagents, these compounds stabilize transient protein–protein interactions by the formation of covalent bonds under physiological conditions. But they also allow for the unambiguous identification of the spatially distributed peptides of the interacting proteins, based on the presence of characteristic reporter ions in the mass spectra that are representative for intramolecular or intermolecular cross-links. Liu *et al.* showed a good correlation between protein three-dimensional-structure data obtained from chemical cross-linking MS (XL-MS) using MS-cleavable reagents and data from cryo-electron microscopy (cryo-EM) on purified protein assemblies. In addition, they also identified in HeLa cell lysates cross-links from protein domains lacking X-ray or cryo-EM three-dimensional-structure data,[37] illustrating the great potential of this strategy in identifying protein–protein interactions. To detect protein–protein interactions by XL-MS, it is mandatory that the amino acids in the interaction area are reactive with the cross-linker.

Secreted proteins play an essential role in communication between immune cells. The immune secretome consists of a wide range of cytokines such as tumor necrosis factors, interferons and interleukins. Multiplex immunoassays and ELISA methods target predefined proteins from the secretome, based on cytokine-specific antibodies. In contrast, newly developed MS-based proteomics methods like BioOrthogonal Non-Canonical Amino acid Tagging (BONCAT) can target the full repertoire of secreted proteins, although limited to *in vitro* experiments only. In a BONCAT assay, an amino acid is replaced by an azido-containing analogue (e.g. methionine replacement by azidohomoalanine). The azido moiety allows for purification of excreted, low abundance, newly synthesized proteins from cell culture medium by click-

chemistry using alkyne-functionalized beads.[38,39] The amino acid modification does not affect the structure and function of the protein, nor does it alter the protein synthesis rate or cell viability.[40,41] In combination with multiplexing technologies based on Tandem Mass Tagging (TMT), N,N-dimethyl leucine (DiLeu) tagging or neutron-encoded reagents, up to 25 samples can be multiplexed in a single run, for unbiased identification and relative quantification of de novo-synthesized proteins in the secretome.[42,43]

Limitations of MS-based proteomics lay in the fact that, like transcriptomics, it is dependent on the availability and quality of annotated protein databases, e.g. number of annotated genes or proteins. The sensitivity of the current generation of mass spectrometers is high, but could improve further to reach the levels achieved with, for example, quantitative polymerase chain reaction. However, as described here and by others,[44] MS is currently very useful for the characterization of a wide range of immunological mechanisms in the context of infection and vaccination.

Miscellaneous techniques

The Systems Vaccinology approach is not limited to the common application of transcriptomics and proteomics and novel techniques and applications are more often applied (Table 1). For example, metabolomics is a high-throughput technology to study metabolic pathways in biological systems. The metabolome covers all small molecules and intermediates (usually < 2000 dalton (Da) molecular weight) that are highly dynamic in the catabolic and anabolic processes of cells. In many immunological studies, metabolomics is now an integral part of system vaccinology, to predict biomarkers and to evaluate vaccine efficacy.[45,46] Untargeted metabolome analysis requires many different sample preparation procedures and analytical tools to identify all metabolites. Gas chromatography, liquid chromatography, capillary electrophoresis, MS, Fourier transform infrared spectroscopy and nuclear magnetic resonance are frequently used or combined to study the metabolome, although with limited precision and accuracy. Improved accuracy, precision and specificity of these assays can be obtained by using semi-targeted or targeted metabolomics strategies if the chemical identities of the analytes are known beforehand.[47]

Humoral and cell-mediated responses

In the context of systems vaccinology, conventional assays that are neither high throughput, nor unbiased, remain necessary to confirm hypotheses. Histology is still the common method to confirm the influx and the location of immune cells in tissues as a result of an infection or immunization. However, visualization of cells in tissue

sections continually improves by better immunoimaging techniques[48] such as confocal microscopy. Ex vivo, assays such as ELISA and flow cytometry are applied to identify and quantify specific cell subsets as well as their activation state and function (such as cytokine production or proliferation). These immunological techniques for studying these responses have also become more powerful. Flow cytometry equipment has improved, for example, by combining the measurement of the amount of proteins per cell (flow cytometry), with visualization of their localization within the cells (microscopy), so-called imaging flow cytometry.[49] Also, more fluorescent labels have become available, increasing the number of markers that can be investigated simultaneously. Multi-parameter flow cytometry enables the identification of these specialized, often rare, subsets of cells.[50] For example, innate cells that play a crucial role in pathogen recognition and in influencing the magnitude and direction of adaptive immunity, such as specific subtypes of antigen-presenting cells and innate lymphoid cells. With respect to adaptive immune responses, the ability to identify rare subsets mainly focuses on antigen-specific B-cells[51] and T-cells,[52] both circulating and tissue-resident cells, and precursors as well as effector and memory cells.[7,8] Cytometry by Time-of-Flight mass spectrometry (CyTOF)[53] uses rare-metal isotopes instead of fluorescent labels for labeling, thereby almost abolishing the spectral overlap seen with fluorescent dyes and expanding the number markers that can be measured simultaneously even further. For example, Van Unen et al.[54] identified 142 immune subsets in the human mucosal immune system.

In addition, the opportunity of sorting specific subsets of cells not only as a population, but also as single cells, in combination with high-throughput analysis of the isolated cells, added yet another dimension. Lymphocyte epitope specificity necessarily is extremely diverse to fight the wide array of possible pathogens and is accomplished by randomness through several recombinatorial events and additional random changes during lymphocyte ontogeny. For example, the number of different antibodies human B-cells can produce, outnumbers the B lymphocytes (~10^{11}) in the human body.[55] High-throughput single-cell analysis enabled detailed interrogation of these broad B-cell and T-cell repertoires.[56] These techniques also revealed that priming might not always be predictive, as preferential expansion of B-cells and T-cells also plays an important role.[57] This illustrates that enhanced understanding of lymphocyte responses not only leads to identification of novel early markers for efficacy of vaccines, but is also useful for their evaluation. Hypotheses with respect to B-cell and T-cell epitopes can be derived from MS-based studies as described in the former paragraph. Moreover, current knowledge with respect to HLA specificity has also enabled the prediction of T-cell epitopes in silico, decreasing the time needed for analysis.[58] As B-cell

epitopes are often conformational, current knowledge does not yet enable *in silico* predictions to the same extent as for T-cells. For B-cell epitope analysis of complex multi-protein vaccines, antibody–antigen binding can be investigated using two-dimensional gel electrophoresis with Western blotting to pinpoint immunogenic antigens.[59] Subsequently, peptide arrays can be applied to investigating the immunodominant epitopes of these discovered antigens.[60]

The ability to isolate single antibody-producing B-cells and subsequent DNA sequencing of immunoglobulin genes in high-throughput settings (Ig-seq) has greatly impacted the depth of understanding with respect to the antibody repertoire.[55] Single-cell sorting in combination with B-cell cloning has enabled the isolation of specific antibodies able to neutralize pathogens, including rare antigens with characteristics that are of special interest, such as antibodies that are broadly cross-reactive among highly variable viruses by binding to conserved regions. This was, for example, found for influenza hemagglutinin (HA) subtypes upon infection as well as vaccination.[61–64] In general, this has shifted the focus from the most immunogenic epitopes to the more conserved regions. However, it has proved challenging to design vaccines that induce strong responses against these regions. Challenges with respect to design of vaccines inducing such broadly neutralizing antibodies for HIV and influenza virus were extensively reviewed by Corti and Lanzavecchia.[65] For example, other regions might need to be removed or de-immunized and the conserved epitopes might not be easily accessible, preventing the vaccine-induced antibodies from binding to the natural target. Another point of attention is that a focus on increased immunogenicity should not lead to a decrease in safety. For example, antibodies with long or charged CDR-H3 regions enable binding to occluded sites on pathogens and mediate pathogen neutralization but are also more likely to be autoreactive.[55]

IgG antibody levels have served as correlates of protection for traditional vaccines,[66] but in the context of systems vaccinology, not only antibody quantity, but also functionality is increasingly investigated. Antibodies may inhibit other functional antibodies or enhance disease by facilitating virus entry into cells. However, non-neutralizing antibodies may still be functional and could have more impact on protection from disease than neutralizing antibodies, for instance by interaction of the antibody constant domain with Fc receptors. This interaction is determined by two characteristics, antibody subclass (irreversible) and antibody glycosylation (fast and reversible) that can for example impact the affinity for Fc receptors.[67] The glycosylation profile of antigen-specific antibodies is changed upon vaccination, whereas the glycosylation profile of total IgG is not affected. Interestingly, despite very different pre-vaccination glycosylation levels in populations in different parts of the world,[68]

profiles induced by vaccination are similar, as was shown after influenza vaccination.[69]

However, different vaccine formulations can induce different glycosylation profiles.[68] This is likely also influenced by the adjuvant present in the formulation, either directly or via cytokine induction.[70] Though the biological implications of these differences are not yet clear, the recent developments in systems serology demonstrate that multiple parameters of the antibody response, such as IgG subtype and antibody glycosylation among others, influence antibody functionally and therefore vaccine efficacy.[71–73]

Data analysis and bioinformatics

One of the difficulties in systems approaches is the amount of data. Statistical evaluation, visualization and extracting meaningful conclusions are major challenges and require multiple tools and expertises.[74,75] New technologies in systems vaccinology such as metabolomics may require different statistical and bioinformatic analysis strategies compared with transcriptomics and proteomics data sets, as described by Ren *et al.*[76] Visualization of data from full genomes or proteomes was initially performed with tools applying e.g. heatmaps, Venn diagrams and network analysis, e.g. with Cytoscape.[77] These allowed visualization of numerous individual data points or groups, but also the clustering of related data points. Additionally, the generation of multiple systems-scale data sets enables the comparison of immune responses induced by different vaccines in an unbiased manner.[78,79] Hierarchical Stochastic Neighborhood Embedding (HSNE) enables us to visualize data of cellular composition of millions of cells in detail up to the single-cell level.[80] Although these graphs provide an easier perspective on trends in the data sets, they are not designed to provide information on the biological function of individual data points. The development of data mining tools (Fig. 1) has given scientists a handle to translate their data sets into the description of relevant biological processes.[81] This allows for investigation of the involvement and mutual interaction of pathways, processes and cell types. Tools that are focused on pathway analysis include DAVID,[82] Pathway-Express,[83] Gene Onthology (GO)[84] and the Kyoto Encyclopedia of Genes and Genomes (KEGG).[85] With BioGPS, the involvement of cells or the different stages in cell activation can be investigated.[86] This has also led to the development of specialized databases for specific research topics. For example, InnateDB contains data on innate immune responses[87] while INTERFEROME focuses on interferon-related processes[88] and the CEMiTool can be used for co-expression analyses and discovery of functionality of genes.[89] For the discovery and analysis of the repertoire of T- and B-cell receptor sequences, VDJviz can be applied for the routine

analysis and quality control of sequencing of immune repertoires[90] and VDJdb is designed for the annotation of T-cell receptor repertoire data.[91] These databases evolve and expand continuously based on novel data, scientific insights and technologies. Depending on the choice of data mining tools, the outcome of results might differ. However, within the Human Vaccines Project,[92] researchers demonstrated that there was no difference in the outcome of their data when it was either analyzed using a tool based on prior knowledge, in this case the NetAnalyst platform, or with an unbiased tool, DIABLO.

In addition, to generate knowledge and understanding, large data sets are used in computational systems biology studies to predict host immune responses.[75,93] From the antigen point of view, machine-learning tools are widely applied to predict B-cell[94] and T-cell epitopes[95] and novel tools such as the IL17eScan[96] enable us to predict the capacity of epitopes to induce interleukin-17. All these data can be gathered *in silico* prior to or simultaneous with laboratory studies to confirm the role of host biomarkers and antigen epitopes in vaccine responses.

Application of systems vaccinology in vaccine research

In each phase of vaccine development (Fig. 2), systems vaccinology techniques can be applied to improve the quality of the vaccine development process as well as decreasing the risk of late-stage failure as a result of increased knowledge (Table 2). Apart from host–vaccine responses, a systems approach can also provide essential information on the vaccine itself, more specifically the antigen composition. Most vaccines belong to one of four groups: (i) live-attenuated, (ii) whole inactivated, (iii) subunit and (iv) conjugate vaccines. Detailed vaccine characterization is mainly of interest for complex multi-protein vaccines such as live-attenuated and inactivated (bacterial) vaccines if for instance batch consistency must be proven.

Exploratory studies in vaccine development

During the discovery phase a systems approach can be implemented to investigate vaccine composition as is especially interesting for complex multi-protein vaccines (Fig. 2). For example, transcriptomics and/or proteomics approaches can be applied during strain generation for an inactivated vaccine to provide detailed information on protein composition and, more specifically, the proportion of immunogenic proteins. These techniques can subsequently be used for process development to verify whether or not the product is similar when produced at a larger scale. This was, for instance, demonstrated for pertussis whole-cell vaccines.[97] Information on the relationship between cultivation conditions and the composition of the final vaccine, considerably enhances the process of

optimizing vaccine efficacy. In addition, it reveals key process parameters that can subsequently be tightly monitored, making the process more consistent and thereby eventual vaccine release easier.

To study the vaccine responses in the discovery phase, systems vaccinology methods can be used to study the mechanism of action of vaccines or adjuvants, both *in vitro* as well as in experimental animals (Fig. 2). *In vitro* cell-based assays allow the gain of information on the magnitude and direction of the innate response, but they lack the possibility to study coherent interactions between innate and adaptive immune responses. Especially in the transition from innate to adaptive responses, a lot of immunological information is still missing.

While the main focus in systems vaccinology is on vaccine-induced responses, pathogen-induced responses during infection are also of great interest, as these can enable the discovery of potential markers of, or mechanisms involved in, protective immunity, or unwanted immunological effects such as immune evasion (Fig. 2). Systems biology can assist to investigate host–pathogen interactions by looking at pathogen behavior and clearance after infection. A natural infection sometimes induces effective protection, e.g. mumps, that may serve as a benchmark for a vaccine, whereas other pathogens may display immune evasive or suppressive responses, which one would like to avoid in a vaccine-induced response. A systems-based approach was for instance used to investigate infection-induced responses during sepsis.[98] In terms of vaccine-preventable diseases, investigators have looked at infection-induced responses for pathogens such as RSV,[99–101] *M. tuberculosis*,[102] *B. pertussis*[12,78,103] and influenza.[104,105] The course of a *B. pertussis* infection was investigated in mice to unravel molecular and cellular signatures of the effective infection-induced immunity.[12,106] Results indicated that a *B. pertussis* infection induced T helper type 17 (Th17) cells and mucosal IgA responses that were preceded by a prolonged acute-phase response, broad pathogen recognition and early gene signatures of subsequent T-cell recruitment in the lungs.[12] In addition, the infection led to enhanced activation of pulmonary innate immune cells and markers indicating recruitment of CCR6+ B-cells and Th17 cells.[106] These promising markers of infection-induced responses should ideally be confirmed in human *B. pertussis* challenge models.[107] In a comparative network analysis of two data sets, the pulmonary molecular signatures that preceded the effector cells in *B. pertussis* infection-induced-immunity[12] were compared with signatures induced upon pulmonary immunization with a novel Outer Membrane Vesicle Pertussis vaccine (omvPV).[15] Network analysis demonstrated that similar genes and pathways were involved in both responses but that the intensity and kinetics between infection-induced and vaccine-induced responses were different. Both responses did, however, result in induction
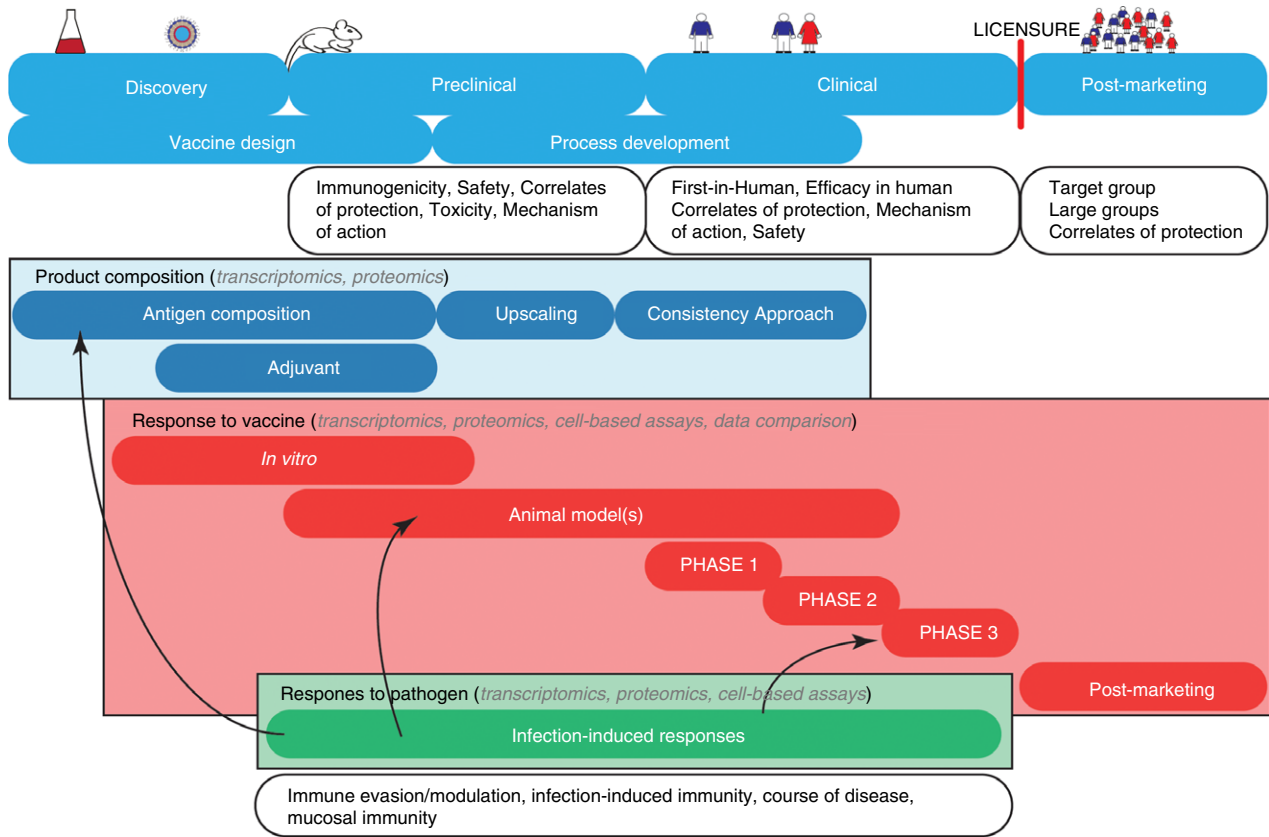
Figure 2. Vaccine development and application of systems vaccinology. Systems vaccinology can be applied to all phases implementing different techniques (e.g. transcriptomics, proteomics, cell-based assays) to provide detailed insight in different research objectives, such as composition, immunogenicity and safety of the vaccine. The host–pathogen responses are not essential for vaccine development but since protection after infection is often superior in terms of efficacy – but not safety – this knowledge can be useful throughout the vaccine development chain, as illustrated by arrows.

Table 2. Applications of systems vaccinology

Aim, deliverables	Benefits	Examples
Predicting responses	Clinical development, post-marketing surveillance	2, 3, 14, 120, 121, 122, 123, 124, 125, 126, 129, 175
Understand mode of action of vaccines	Risk mitigation: less late-stage failure	127, 129, 176
Identify universal vaccine signatures	Improve vaccine development	79, 118, 133
Select new immune modulators and delivery systems	Risk mitigation: less early-stage failure Better vaccines	13, 111, 113, 128
Assess vaccine safety and adverse effects	Clinical development, post-marketing surveillance	117,118,119
Understand mode of action of infection	Host–pathogen interaction	100, 102, 104, 105
Rational vaccine design	Risk mitigation: less early-stage failure Facilitates regulatory acceptance	N.A.
Development of animal models	Improved early development Risk mitigation: facilitates preclinical to clinical decision.	118

of mucosal immunity and better protection compared with subcutaneous vaccine administration.

Systems approaches have also been used to investigate innate responses, for example responses induced by adjuvants.[108] *In vitro* studies were performed to investigate monocyte-specific responses induced by alum salts using a proteomics approach[109] and to study subset-specific dendritic cell responses following lipopolysaccharide stimulation using a transcriptomics approach.[110] These extensive *in vitro* studies provide insight into novel markers, pathways and cell subsets activated by adjuvants. Yet the complexity of cell-to-cell interaction during immune

responses requires *in vivo* studies. This was also addressed by Mosca *et al.*,[13] who conducted one of the first systems-based studies in mice for better understanding of adjuvants, such as MF59, alum, CpG ODN and α-GalCer. Adjuvant-induced responses were profiled in muscle at the site of injection. This provided comparative information on plain adjuvant responses such as the fact that CpG elicited stronger systemic innate responses compared with MF59 and alum. Others have studied the responses of adjuvants in combination with antigen, that might also influence the immune response. Olafsdottir *et al.*[111] determined molecular signatures induced in mice of four clinically tested vaccine adjuvants (CAF01, IC31 GLA-SE and Alum) using a tuberculosis vaccine candidate (H56) as model antigen. With a transcriptomics analysis, signatures of T follicular helper (T$_{fh}$) and germinal center (GC) B-cell responses were found to be enhanced in adjuvated vaccines compared with those of the H56 antigen alone. Using a similar combination of H56 adjuvanted with CAF01, Santoro *et al.*[112] applied systems vaccinology to demonstrate that recall innate responses following boosting were modulated by the use of adjuvant in the primary immunization. Lindqvist *et al.*[113] focused on specifying the molecular signatures in mice induced by mucosal adjuvants for vaccination against sexually transmitted infections.

Relevant animal models to study infection are not always available. In such cases, the application of systems approaches in vaccination studies can provide valuable information on the mechanism of action of candidate vaccines. Gómez *et al.* unraveled transcriptomic activation markers of human myeloid and plasmacytoid dendritic cells upon stimulation with a vaccine candidate against HIV/AIDS, based on an MVA-based vector. Subsequently, HIV-1-specific CD4$^+$ and CD8$^+$ T-cell memory responses were demonstrated *in vivo*.[114] Strouts *et al.*[115] were able to link early specific gene transcripts related to T-cell activation and type I interferon response to the magnitude of neutralizing antibody titers after immunization with a live-attenuated tetravalent dengue vaccine in non-human primates. As part of the development of a novel Outer Membrane Vesicle Pertussis vaccine (omvPV), vaccine-induced responses were investigated in mice. An extensive proteomics approach for antibody profiling was used to determine the distinct antibody specificity and subclass distribution induced by different pertussis vaccines.[59] Transcriptome and cytokine profiling demonstrated reduced pro-inflammatory responses, potentially leading to less adverse effects by the omvPV compared with a classic whole-cell pertussis vaccine while maintaining the vaccine efficacy.[116] To investigate the effect of the route of immunization on protective immunity, the responses induced by subcutaneous and pulmonary administration of omvPV in mice were compared.[15] The study demonstrated improved mucosal immunity, and

therefore protection, against *B. pertussis* after pulmonary immunization. Finally, in terms of vaccine safety, Mizukami *et al.*[117] applied the systems-approach in a rat model to predict safety and batch-to-batch consistency of influenza vaccines. Wang *et al.*[118] investigated blood gene signatures in non-human primates immunized with seven marketed or experimental vaccines to isolate markers of vaccine efficacy and safety. Whereas overlapping trends in markers of efficacy were found between some vaccines, no markers were found correlating to adverse effects.

Clinical studies

Predictive markers that have been found in the exploratory studies (described in the previous section) can be applied in subsequent clinical studies. Moreover, systems vaccinology analyses are also useful in a clinical study itself to obtain better insight into safety and efficacy (Fig. 2). However, to our knowledge in only a few clinical studies has systems vaccinology been performed to assess signatures that correlate to adverse effects, such as fever and seizures. In the public–private project BIOVACSAFE, systems approaches are applied to investigate markers correlating to vaccine safety in human and animal models potentially providing novel markers.[119] Investigators have conducted systems-based studies in humans investigating vaccine candidates against Ebola (rVSV-ZEBOV),[120] HIV-1,[121] malaria (RTS,S)[122,123] and *M. tuberculosis* (M72/AS01).[124] For Ebola (rVSV-ZEBOV), IP-10 and CXCR6$^+$ natural killer (NK) cells were independently correlating with antibody titers, suggesting that IP-10 could be a potential target to influence vaccine-induced responses.[120] Anderson *et al.*[121] investigated transcriptome profiles in the blood of healthy volunteers following immunization with a novel HIV-1 vaccine adjuvanted with a Toll-like receptor 4 agonist. High responders, based on serum antibodies, contained modules of genes expressed in NK cells, whereas modules of genes related to myeloid cells, monocytes and integrin cell surface interactions were detected in low responders. These signatures enable vaccine responses to be distinguished in an early phase and perhaps even allows steering of immune responses in low responders by adjustment of the vaccine. In a phase I randomized controlled trial using AS03-adjuvanted and unadjuvanted inactivated split-virus H5N1 influenza vaccines, the effect of adjuvant on vaccine-induced responses, such as serum cytokines, antibody titers, and gene expression levels, was investigated. This led to novel insights into antigen processing, presentation markers in neutrophils and revealed the correlation of serum IP-10 levels with proliferation of NK cells.[125] Interestingly, for the prediction of protection induced by malaria (RTS,S) immunization, NK cells correlated negatively whereas positive correlations were detected for molecular signatures of B-cells and plasma cells[122] and the nuclear

factor-κB and interferon-γ pathways.[123] These findings indicate that different vaccine–adjuvant combinations may induce different immune (non)effective responses.

Post-marketing studies

After vaccines have been licensed and introduced on the market, post-marketing surveillance studies are performed to monitor vaccine safety and efficacy in larger populations (Fig. 2). At this point in the vaccine life cycle, correlates of protection, or surrogates thereof, are sometimes known, however the mechanism of action is often underexposed. Systems approaches in this phase are not routinely applied, as far as we know. The validation of the methods is complex and expensive, with the added value not yet proven convincingly. However, pioneering studies have demonstrated that vaccine efficacy after yellow fever and influenza vaccination could be predicted in an early stage after immunization by analyzing molecular signatures.[14,126,127] For the yellow fever vaccine, the enhanced levels of specific gene expression profiles in blood obtained 1 to 7 days after vaccination were predictive for antigen-specific CD8$^+$ T-cell levels and antibody titers usually obtained 60 days after immunization, thus inducing a significant time advantage. In a clinical phase, regulators might want to see positive classical end-points but for post-marketing purposes this approach of looking at early correlates may be valuable. Furthermore, these studies are of value in comparative studies of different vaccine formulations. Nakaya *et al.*[128] compared two different vaccine formulations, trivalent inactivated influenza vaccine (TIV) and MF59-adjuvanted TIV (ATIV), in children and predicted influenza antibody titers 1 month after vaccination with a seasonal influenza vaccine across five consecutive seasons by analyzing specific signatures of innate immunity and plasma blasts.[129] In addition, a systems biology approach could also be applied to investigate the differences in vaccine responsiveness in different target groups. For example, researchers predicted age-related vaccine hypo-responses against a hepatitis B vaccine in the elderly with a gene marker[130] and exposed transcriptomic signatures of vaccine-induced immunity, both cellular and humoral immune responses, after seasonal influenza vaccination specifically in older adults.[131] Obviously, these post-marketing surveillance studies facilitate further clinical development of improved vaccines or provide insight into general mechanisms that will enhance future vaccine development, even if correlates of protection are unknown.[132]

Finally, a great benefit of systems vaccinology studies is the ability to combine data sets from vaccine responses against different pathogens in integrative network modeling to reveal detailed insight into universal signatures of vaccine responsiveness.[78,79,118,133] Li *et al.*[79] compared molecular signatures, induced by five different human

vaccines against specific bacterial or viral infections, which could predict antibody responses. This indicates that investigating responses against a single pathogen can lead to universal markers that serve as benchmark for vaccine development against new emerging diseases. This also supports the desire for data obtained in systems-approaches to be publicly available (open access) for future large meta-analyses.

Future outlook

Systems vaccinology has been implemented in the different phases of the vaccine development chain and has become a relevant approach in the field of vaccinology. The recent progression in systems vaccinology has led to successful initiatives such as the ADITEC project[134] that provided a novel gene expression method to analyze biomarkers of tuberculosis pathogenesis[135] as well as a study where safety and immunogenicity of a novel vaccine against tuberculosis (VPM1002) were evaluated.[136] To achieve maximum vaccine efficacy and safety, future vaccine research will have to focus on target groups such as neonates[137,138] or even on the development of personalized vaccines.[139] Systems vaccinology approaches can be further applied to investigate the inter-group differences in vaccine responsiveness between healthy adults and specific target groups such as infants, and elderly or immune-deficient individuals.[140]

Investigating vaccine-induced responses with systems vaccinology has led to a trend of gathering massive amounts of data going as deep as investigating every molecule in single cells. Acquiring large data sets is far easier than extracting novel relevant information from those data. Furthermore, the amount of data is constantly increasing, especially when combining data sets of different origin (genes versus proteins versus cells). This requires equally advanced tools for (i) data visualization and (ii) translation of content to knowledge and understanding, forcing a dialogue between vaccine developers and bioinformatics experts. In the future, implementation of artificial intelligence could assist in data processing and for data visualization online publications may become interactive allowing readers to scroll through, for example, the immune responses induced by immunization over time.

In summary, systems vaccinology is becoming a valuable tool in each phase of the vaccine development chain. The link with predicting immune responses beforehand is, however, still difficult. Small but important steps are currently taken towards application of the insights created by systems vaccinology. Recent studies show that the application of systems vaccinology is especially interesting for investigating complex or dangerous pathogens such as Ebola virus, HIV, malaria-causing *Plasmodium* spp. and *M. tuberculosis* where phase 3 studies are challenging or even impossible,[120–124] or even for investigating the

heterologous or non-specific effects that vaccines induce against other diseases.[141] These promising changes call for the inclusion of systems vaccinology as early as possible in the vaccine development chain to better understand why some vaccines work and others do not. This will enable efficiency of vaccine development proportionally in the design phase and will lead to improved vaccine evaluation in early phases, thereby reducing time and costs.

Author contribution

R.R., E.R., H.M., B.M. and G.K. wrote the review.

Disclosures

The authors declare no competing interest.

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