

Systems immunology: just getting started

Mark M Davis^{1,2}, Cristina M Tato² & David Furman^{2,3}

Systems-biology approaches in immunology take various forms, but here we review strategies for measuring a broad swath of immunological functions as a means of discovering previously unknown relationships and phenomena and as a powerful way of understanding the immune system as a whole. This approach has rejuvenated the field of vaccine development and has fostered hope that new ways will be found to combat infectious diseases that have proven refractory to classical approaches. Systems immunology also presents an important new strategy for understanding human immunity directly, taking advantage of the many ways the immune system of humans can be manipulated.

A systems approach to an area can be defined as a broad strategy for understanding how a complex set of interacting components works to produce certain outcomes. Long ago, prescient immunologists coined the term ‘immune system’ in recognition of just how well this label fits the field, but for much of its long history, most insights into the immune system came from painstaking examination of the parts and their largely individual properties and not so much of activities across the whole system. This is understandable, as there are more parts to the immune system than even the reputed 400 cheeses in France, with 350 CD (‘differentiation cluster’) antigens, over 100 cytokines and chemokines and at least that many cell subsets, and thousands of genes. This is daunting, to say the least, but advances in technology have now made it feasible to measure a good swath of this complexity in a relatively workable way. This has resulted in the discovery of new relationships and participants in immunology, insight into why some vaccines are more effective than others, and important connections with human diseases and mouse models of disease. These data are also now being used to model important aspects of the immune response, and this will be one of the most important goals.

Two main types of systems immunology can be seen in the literature, one of which uses this approach to elucidate signaling pathways that are important in the immune system^{1,2}. This has resulted in a new understanding of these pathways in general and the creation of useful models of how different cells of the innate immune system function. These approaches have been very capably reviewed elsewhere³ and will not be discussed here. Instead, here we describe another type of systems approach to immunology that focuses on the cells and the molecules they use for communication and how they respond to vaccines, infections and so on. The reason for this is that the main effector activities of the immune system are mediated by specialized cells

that communicate with each other and various tissues via cytokines and chemokines. These cells are relatively autonomous, and their only means of sensing what they need to do comes through their various cell-surface receptors. They can be mobilized *en masse*, but each and every cell must receive the correct signals. Thus, specialized cells are the principal unit of activity in the immune system. A CD4⁺ T cell can detect even a single complex of peptide and major histocompatibility complex (MHC) on another cell, and it serves as the detector, the signal integrator and the effector (in this case, by secreting cytokines)^{4,5}. The more of a particular antigen that comes into the system, the more T cells with that specificity are recruited. Since white blood cells that make up the bulk of the response repertoire are largely mobile, with the exception of resident memory cells (which were once mobile), it is reasonable to think that they are all autonomous. Fortunately, the technology now exists to measure, *en masse*, most of the known cell types, their state and function, and their products (including cytokines, chemokines and metabolites) and the genes encoding those molecules^{6,7}. This has led to a major change in how research is performed in many laboratories, since it is now feasible to use high-throughput and high-bandwidth methods for unbiased exploration of a variety of immune responses, which has revealed key participants that contribute to health and disease, and to generate new hypothesis and mechanistic studies (Fig. 1). This ability to generate large data sets has evolved together with technological advances in informatics and statistics that have now made it feasible to generate integrative models of the human immune response^{6–8}. Thus, a systems approach with the objective of measuring the responses of much of the immune system comprehensively is quite feasible and is the principal subject of this Review. This is not meant to supplant traditional forms of enquiry in immunological research; indeed, as new phenomena are discovered, each must be carefully investigated down to its specific mechanism, and typically the tremendous power of the mouse system is the best way to do that, if the initial data are from studies of humans. So what do researchers hope to achieve with this type of systems immunology? First, it can be seen as a logical evolution of the field from an intense study of its parts to a broader and more integrated view of how those parts work together to produce particular results. Furthermore, it can reveal (and has revealed) unexpected examples of higher-order

¹Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, California, USA. ²Institute for Immunity, Transplantation and Infection, Stanford University School of Medicine, Stanford, California, USA. ³Institute for Immunogenetics, Jose de San Martin Clinical Hospital, National Scientific and Technical Research Council, Buenos Aires, Argentina. Correspondence should be addressed to M.M.D. (mmdavis@stanford.edu).

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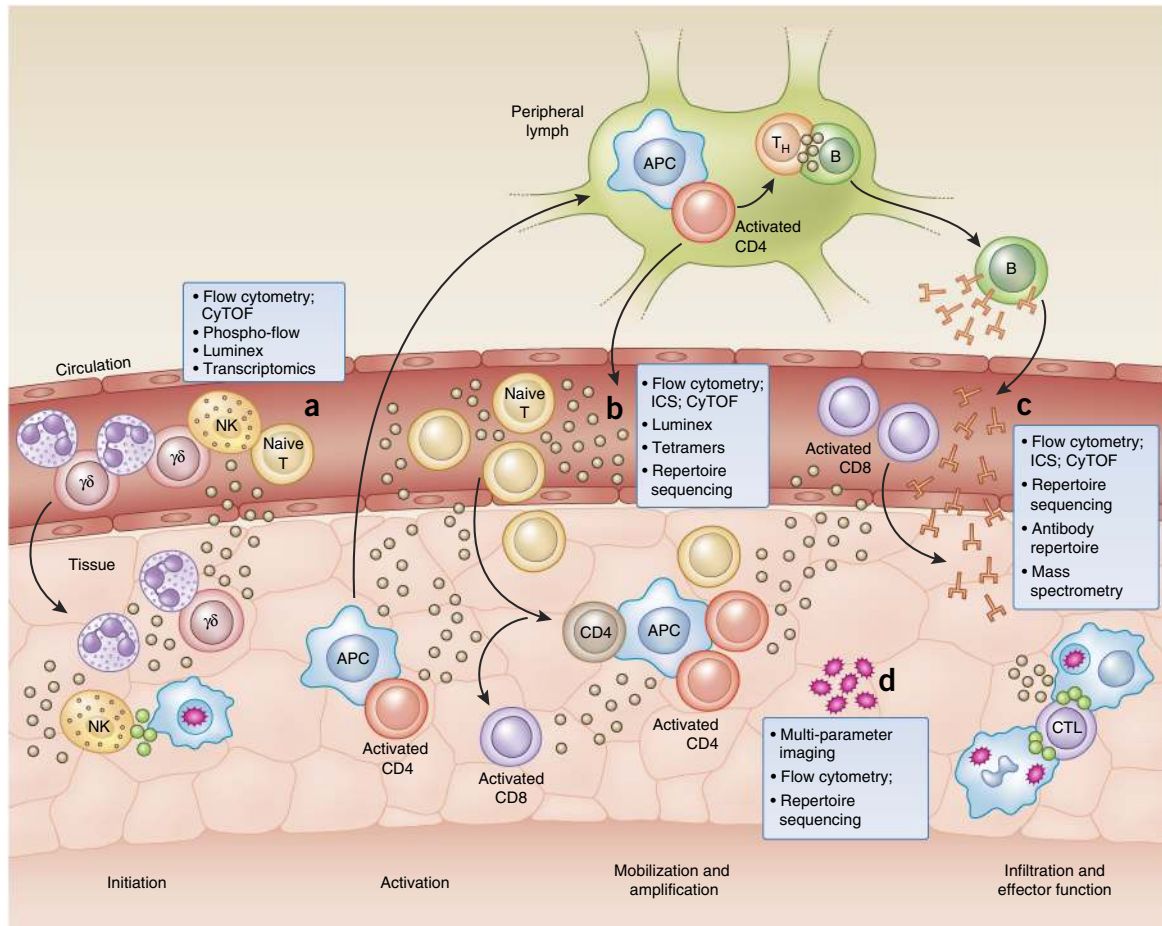


Figure 1 Tools of the trade: a systems-immunology view. Investigating the immune system can be accomplished with a variety of techniques applied to the analysis of peripheral blood samples obtained from healthy donors, as well as those obtained at specific time points during infection, disease or after vaccination. Innate cell subsets can be characterized by RNA-seq, flow cytometry, cytometry by time-of-flight (CyTOF) and flow cytometry of phosphorylated proteins (Phospho-flow) to reveal mediators of early inflammation, and their signaling pathways (a). Cells can be classified by phenotype and functional capacity on the basis of a variety of cell-surface markers and receptor expression. Effector proteins, such as cytokines and cytolytic granules, along with other products of activated cells, can be detected intracellularly by flow cytometry or in the serum with other methodologies, such as bead-based multiplex assays (Luminex) or immunoassay kits (from Mesoscale Discovery), to rapidly survey more than 100 analytes in a single, small sample (b). Cell subsets of the adaptive immune system can be investigated for antigen specificity by tetramer technology and mass spectrometry to explore peptide presentation, as well as sequencing of the TCR and BCR repertoire for the identification of clonally expanded populations. Furthermore, antibody-repertoire sequencing can be used to characterize the extent of isotype class switching and affinity maturation (c). Many of these techniques are currently being adapted for the analysis of smaller tissue-biopsy samples to extract information about the local inflammatory environment and the infiltrating cell populations (d). s APC, antigen-presenting cell; T_H, helper T cell; B, B cell; CD4, CD4⁺ T cell; NK, natural killer cell; γδ, γδ T cell; T, T cell; ICS, intracellular cytokine staining; CD8, CD8⁺ T cell; CTL, cytotoxic T lymphocyte.

cooperativity between those parts, both cell types and pathways, that have provided a more complete idea of what is actually going on. Second, and perhaps at least as important, since cells and molecules and gene expression can be measured accurately in a blood sample (Fig. 1), it is providing a powerful view of the human immune system, which is much needed, especially because most of the methodologies now used for mouse immunology are not typically possible in human research. In contrast to the focus of most mouse immunology, there is a strong focus on blood in human studies, as this can be obtained easily from almost any willing human, healthy or not, and while the circulatory system is not an immunological organ, it is the conduit by which almost all important immunological traffic occurs. Being able to apply this to humans also provides the advantage of being able to assess the consequences of the thousands of immunological perturbations that humans are exposed to, meaning every vaccine, infectious disease, autoimmune disease, immunomodulatory treatment

and so on. More broadly, it is helping researchers to overcome the inherent limitations of a field that has relied largely on a single experimental system, inbred mice, for which normal genetic variation has been eliminated and very few environmental influences are permitted. While such compromises were and are critical to solving key problems, they are, nonetheless, compromises, and their effects need to be considered and accounted for. This is where humans fit in perfectly. Humans are largely outbred and are exposed to myriad environmental stimuli with broad variations across the globe. That has resulted in the emergence of many important discoveries about basic immunology via human studies, such as the observation that negative selection is not nearly as efficient as thought on the basis of classic mouse experiments dating back 25 years⁹, and thus self-specific T cells are very abundant in healthy people, possibly because the threat of infectious disease is much greater from an evolutionary perspective than is the threat of autoimmunity.

The need for teamwork

Perhaps one of the greatest barriers to implementing projects in systems immunology is that a variety of high-level skill sets are needed. Just the fact that a team approach is needed is a major barrier in modern biology, in which researchers have long subscribed to the mythology of the lone hero going into the cave and slaying the dragon. Perhaps that worked in the middle ages, but today's reality is that cooperative approaches are more and more the norm as the complexities of modern work extend beyond even the most talented people. The good news is that most medical research centers have the requisite skills, but there just must be a willingness to work together. The core skills are clinical, technical, bioinformatics and immunological (Fig. 2). Clinical skills are required for the obvious reason that this typically involves live human beings, not to mention the wealth of knowledge needed, especially if the study is about a particular disease and the collection and collation of valuable meta-data from each participant. Bioinformatics, with inputs from computer sciences and statistics, is essential because this systems approach requires the ability to manipulate large data sets to extract meaning, but standardized user-friendly analysis systems are not yet available. Crucial to the success of any systems study is the interaction of bioinformatician and immunologists. Expertise in the field of immunology is needed to direct focus on biologically relevant results obtained from computational analyses, to interpret the mechanistic implications according to immunological theory and, ultimately, to design new experimental questions and models and to introduce translational applications. Finally, technology is a must, because a systems-immunology approach requires instrumentation that can extract large amounts of information rapidly and relatively inexpensively from often fairly small samples. Ideally, this means a core facility with the most-advanced equipment, and especially valuable is one in which there are trained staff to run the assays. This is important not only for reproducibility and reliability but also to free research personnel and trainees to focus on the data. Here at Stanford University School of Medicine we have such a facility, the Human Immune Monitoring Center, which fulfills this role extremely well and has enabled hundreds of studies, mostly but not exclusively human, some large and many small (Figs. 1 and 2). The other aspect of technology that has been especially critical to advancing human studies is the input of research groups and bioengineers who are developing new technologies or who are early adopters, keeping investigators and facilities up to date on which technology is the best to use.

The need for innovative technologies and analyses

Since many of the standard manipulations used for the study of mouse immunology cannot be used in ethical work studying humans, there has been a strong need for technologies that can extract as much information as possible from often very limited clinical specimens, particularly blood. A mainstay here has been gene-expression arrays, which, while not a new technology, when applied to whole-blood analysis, are very 'noisy' because of the wide variation in subsets of human white blood cells. This results in a gene-expression pattern that is more reflective of particular person's cell types rather than differences in the content of mRNA per cell. Another problem is that of 'false discovery', in which the sheer number of genes being surveyed increases the probability of finding a difference that is an artifact. Fortunately, in the latter case, grouping co-expressed genes into modules whose products act in concert provides way to find true changes that is both simplified and statistically valid^{10,11}, and for the former problem, methods have been developed to deconvolute gene-expression data on the basis of minimal information on the frequency of

immune-cell subsets from healthy people and in patients with disease as well^{12–16}. Particularly promising in this context is the CIBERSORT program, which can predict cell frequencies from gene-expression data and has thus opened up the large amount of gene-expression data in the public domain for analysis¹⁷. A third challenge in data science is to deal with so-called 'overfitting', a statistical concept that refers to the propensity of a model to capture 'noise' in the data rather than a genuine relationship, which is often the result of models that are too complex. In such cases, the model learned from a given data set and by a given statistical method will probably fail to generalize to additional data sets. Various methods have been developed to avoid this problem. For example, in regression analysis, regularization methods impose mathematical constraints on the regression coefficients, which enables 'simpler' solutions—in other words, the selection of models with fewer immunological components that are found to be relevant for a given mathematical task, which thus avoids overfitting^{18–20}. Finally, another major analytical effort has focused on deriving key gene 'signatures' from data obtained by microarray or RNA-based next-generation sequencing (RNA-seq) to elucidate key events in human immunity, such as the progression to active disease in tuberculosis^{21,22} or the differences in the immune response to infection with influenza virus versus the response to other viral or bacterial infections²³. Methodologies such as these have great potential to affect clinical diagnostic abilities, as well as the ability to monitor and predict responses to treatment and recovery from disease.

Technological advances have been equally important, and while most are discussed in the context of the sections below, those involving 'deep' cell phenotyping and repertoire analysis have been the most important. In particular, the invention of mass cytometry and its subsequent development^{24–26} was a watershed moment during which research went from 12 colors, with flow cytometry, to 25 and 45 or more labels on single cells; this essentially redefined all the major subsets of white blood cells, given the wealth of data now possible^{27–29}. It can also work on such small numbers of cells (fewer than 1×10^6 peripheral blood mononuclear cells) that studies of samples obtained from pediatric subjects are more easily performed^{30,31}. Additional technological advances have been extremely useful in the functional genomics arena. For example, methods using the CRISPR-Cas9 system, such as Perturb-Seq³², CROP-Seq³³ and CRISP-Seq³⁴, have enabled the investigation of the effect of hundreds or thousands of genetic changes in parallel. By taking advantage of RNA-seq as a 'readout', these approaches overcome the limitations of previous ones and allow the analysis of perturbations and the phenotype of each cell simultaneously.

Vaccination responses and infectious diseases

Responses to vaccination have received the lion's share of attention in these early days of systems approaches because of both the need for more-effective vaccines and the relative ease with which approved vaccines can be administered to human volunteers to probe their ability to elicit an immune response. Vaccines also tend to 'push many buttons' in the immune system, so it has been fruitful to compare different vaccine responses in various cohorts. It even has its own subdivision as 'systems vaccinology'³⁵. The first broad analysis of the effect of a vaccine was research that focused on the vaccine against yellow fever^{36,37}, believed to be one of the most successful in terms of its nearly universal response rate. Those early papers focused largely on transcriptional responses, but subsequent work incorporated more of the breadth of the immune response, including serum cytokines, cell phenotyping and functional immunological assays to study the mechanisms of vaccination against that and other diseases, such as

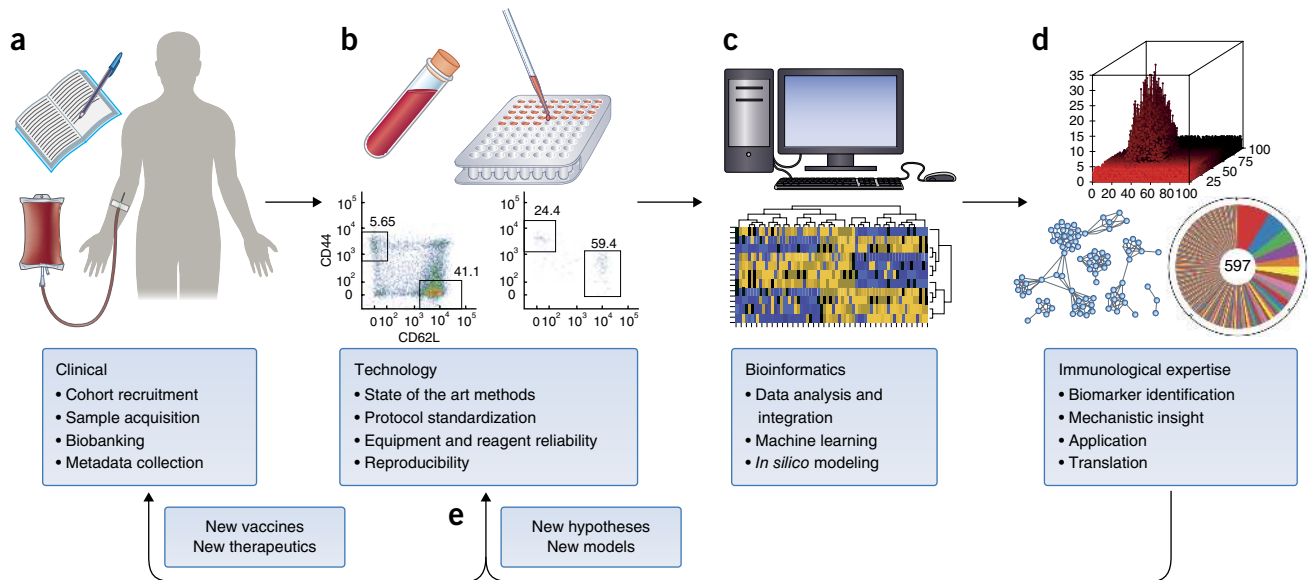


Figure 2 The ideal team. Cross-disciplinary efforts have allowed considerable advances in human medical research. The integration of clinical research (a) was made possible by the invention of new technologies (b) for comprehensive analysis of precious blood and tissue samples. The development of new algorithms for the visualization and analysis of such large data sets (c) has enabled novel insights and the generation of new hypotheses (d). Such collaborations should help to bridge the gap between bench and bedside by accelerating the translational pipeline (e).

pneumonia, measles and influenza^{38–45}. The vaccine against influenza is particularly interesting as a standard vaccine to study, as it has a markedly variable response rate, with ~80% of younger people and less than 30% of older people judged to be stimulated enough for a protective response⁴⁶. That is a dismal success rate, and the reasons for it have been a chief target of many papers in this nascent area. For the RTS,S candidate vaccine against malaria, which confers substantial protection against infection with *Plasmodium falciparum* in humans, research has demonstrated the utility of systems approaches in identifying molecular and cellular signatures associated with protection and immunogenicity. In particular, both the titers of immunoglobulin G antibodies to circumsporozoite protein and the population expansion of circumsporozoite-protein-specific polyfunctional CD4⁺ T cells producing a combination of the B cell-stimulatory molecule CD40L and the cytokines IL-2, TNF and IFN- γ after vaccination are predictive of protection against challenge with the pathogen⁴⁷. Mechanisms reported to contribute to vaccine responses include a failure in the induction of cell death⁴², the metabolism of lipids and endocrine factors that suppress immune-system function⁴⁴, prior exposure to infection⁴⁸, and nutrient-sensing pathways, including the sensor GCN2, which mediates T cell responses to vaccination⁴⁹. There is also a particularly interesting connection to the microbiota, in that stimulation of the receptor TLR5 on B cells in mice by microbiota-derived flagellin is crucial to the maturation of those cells into antibody-secreting cells⁵⁰. Those are among some of the mechanistic insights revealed by systems studies that are helping researchers to consider and weigh the effect of factors that contribute to the variability of individual human responses to vaccination and to holistically incorporate variables derived from the host, pathogen, environment and vaccine (formulation and route of delivery). Furthermore, such systems approaches to studying vaccination responses have great potential to elucidate ways in which existing vaccines can be improved through stimulation of parts of the immune system that are not being stimulated by the current vaccines. There is also the hope that stimuli can be identified that could even generate protective responses to pathogens and diseases

that have so far proven refractory to standard approaches, such as human immunodeficiency virus, malaria, dengue virus or tuberculosis in adults. This has given new life to the vaccine field, which has been facing major challenges with such diseases for which traditional approaches have failed to produce efficacious vaccines.

In addition, longitudinal studies are particularly well suited for inferring the mechanisms of action of vaccines. For example, in a study monitoring the immune system, samples were obtained every 4 weeks for a period of 8 months from patients infected with human immunodeficiency virus who were receiving a therapeutic vaccine; these showed a substantial increase in the number of polyfunctional T cells specific for human immunodeficiency virus, associated with control of viral load⁵¹. In another longitudinal study, samples were obtained periodically from young adults who received the seasonal vaccine against influenza virus, the 13-strain pneumococcal vaccine or a placebo. Gene-expression analysis showed unique and distinct signatures elicited by those vaccines⁵².

Aging

Vaccines in human studies have another use as a general stimulant of multiple components of the immune system for the study of the immunological consequences of various conditions, such as the influence of genetics, neoplasia and so on. This has particular relevance to aging, as there is clearly a sharp drop in the effectiveness of the immune system as most people age, especially over the 65- to 70-year mark. Early investigations noted a sharp increase in the number of CD28⁺CD8⁺ T cells as people age, which correlates with loss of function⁵³, but with the broad approaches now possible, many more aspects of immunity can be surveyed. In particular, there seems to be a loss of the T cell repertoire⁵⁴ that might relate to thymic involution and the subsequent slowing of the production of new T cells to a trickle. However, involution happens in a person's 20s, and repertoire depletion happens many decades later⁵⁵, so is that the real reason? Work on aging has demonstrated strong links between chronic inflammation in the immune system and cardiovascular disease.

A longitudinal study of aging people has shown a widespread elevation of phosphorylated transcription factors of the STAT family, especially in lymphocytes from subjects in their 70s and 80s, which suggests that these lymphocytes had been exposed to inflammatory cytokines⁵⁶. That takes advantage of the fact that lymphocytes are continually circulating throughout the body and thus potentially 'report on' pathologies that might be physically distant. The presence of those hyper-phosphorylated STAT molecules often correlated with an elevation in inflammatory cytokines in the blood and diminished ability of cells from these subjects to respond to cytokine stimulation *in vitro*. That cytokine unresponsiveness was then linked to atherosclerosis and other measures of cardiovascular disease. Another study of that same longitudinal cohort noted, from transcriptional data in over 5 years, that certain gene modules showing enrichment for genes encoding components inflammasomes correlated with older subjects with persistent hypertension and a lack of longevity in their family history⁵⁷. That led to a metabolomics analysis that discovered aberrant nucleotide metabolism in these subjects and two previously unknown danger-associated molecular patterns that stimulated the expression of inflammasomes *in vitro*. In addition, those compounds caused hypertension and infiltration of lymphocytes into the kidneys of mice⁵⁷. This shows how casting a broad net with a systems approach can yield important insight into a common human disease (hypertension) about which very little is known mechanistically. It also adds important specifics about the nature of chronic inflammation that is often seen in the elderly and is linked to many diseases of the elderly, especially cardiovascular disease.

Cancer

While neoplasia takes many forms, the relationship between a particular cancer type and the immune system is clearly a complex and little-understood topic that could benefit from a systems approach. The clinical relevance of having a better understanding is clear, since while new immunotherapeutic approaches involving either checkpoint inhibitors or chimeric antigen receptor (CAR) T cells have beneficial effects, the former has been effective in only a fraction of patients and the latter has not yet been effective with solid tumors. Additionally, better definition of the parameters of anti-tumor immunity will support the more-informed design of vaccines for the induction of clinical immunity to tumor antigens, which has so far fallen short of experimental expectations⁵⁸. The basis of any of these deficiencies is not understood, so better understanding of the immune-system dynamics is clearly needed and could provide the basis for a new generation of anti-cancer vaccines coupled with tolerance-inhibiting agents and/or chemotherapeutics and radiation. Studies aimed at understanding the effects of checkpoint inhibitors, in which a combination of genomic and systems immunology were applied along with bioinformatics analysis, have found that this immunotherapy is associated with T cell responses to mutant tumor antigens that are often reactivated upon treatment with immune-checkpoint inhibitors^{59–61}. Furthermore, systems analysis of a mouse model of cancer has illustrated that multiple components of the immune system are involved and has provided a template and rationale for human studies along these lines⁶².

Autoimmunity

The field of autoimmunity has great potential for systems studies, as clearly in autoimmunity there has been some disturbance to the normally tight regulation of self tolerance, but surprisingly few studies have been done that could be considered systems immunology. One such study has shown that administration of gluten to patients with celiac disease not only induces the expected surge in gliadin-specific

CD4⁺ T cells 6 days after challenge but also induces oligoclonal CD8⁺ and $\gamma\delta$ T cells that appear and disappear from the blood with similar kinetics⁶³. This suggests that there is a complexity to the T cell response in patients with celiac disease that had not been appreciated previously and that this might be a factor in other diseases as well. Particularly relevant in the field of autoimmune diseases, since most of these are sex biased, are the differences in the activity of immune cells in males versus that in females. Applications of systems immunology to this area have just begun, and initial data show gross sex differences for healthy young adults, but for not older adults, in particular subsets of cells, including blood neutrophils, in their maturation status and responses to sera from patients with systemic lupus erythematosus⁶⁴ that were previously unappreciated. In the domain of systemic lupus erythematosus, work has shown that the application of systems approaches to these patients enables accurate identification of molecular networks that stratify disease activity⁶⁵.

Genetics

The widespread use of powerful genetic analyses has resulted in many large studies aimed at finding genes or polymorphisms that underlie various immunological diseases, particularly autoimmunity. While such studies have identified many interesting relevant loci, their contribution to disease risk is typically very small. Also, the role of environmental influences is typically not studied directly. For this reason, we embarked on an analysis of twins, both monozygotic and dizygotic, which concluded that for over 200 immunological traits, including responses to the vaccine against influenza virus, in most cases non-heritable influences were dominant, and that infection with cytomegalovirus had a substantial effect on most of these variables⁶⁶. That supports the proposal that the immune system is (and must be) a highly adaptable system, and not just in the specific T lymphocytes or B lymphocytes that it produces but also in its overall makeup. It is also worth noting that because the immune system utilizes hundreds, if not thousands, of genes, the genetic load is considerable, which means that most people will have some non-functional or poorly functional genes. While this will in most cases be ameliorated by heterozygosity, it could still be deleterious, were it not for the redundancy that is continually seen in the system. This could explain the finding that despite the genetic load, susceptibility to serious infectious disease is very rare, although susceptibility to less-serious versions is not, consistent with published information on this^{67–69}.

BCR and TCR repertoire analysis

One important aspect of an immune response that is just starting to be captured adequately for the purposes of systems immunology is the response repertoire of T lymphocytes and B lymphocytes. This is being made possible by both the rapid progress of DNA-sequencing technology and the development or application of software that is able to capture the diversity or other aspects of B cell or T cell responses. Studies of antibody responses first used high-throughput sequencing to analyze B cell responses^{70,71}. This work was advanced further by the pioneering of single-cell sequencing of tens of thousands of B cells simultaneously⁷². Specific, common immunoglobulin sequence motifs have also been noted that correlate with particular specificities, which indicates that it might be possible to infer the specificity and potential efficacies of a response from sequence alone⁷³.

In the T cell area, the ability of mass cytometry to analyze many labeled molecules simultaneously at the single-cell level led to the description of hundreds of different functional variants of human CD8⁺ T cells, which showed a nearly stochastic array of cytokine combinations²⁸. Combinatorial peptide–MHC staining also has allowed

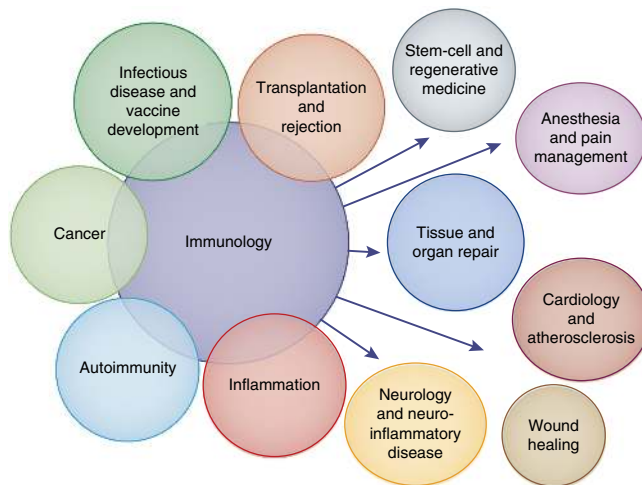


Figure 3 A comprehensive picture of health. The immune system has a fundamental role in a variety of diseases and disorders, including the capacity for resistance, recovery and maintenance of the health of a person.

over 100 different T cell specificities to be surveyed in a standard human blood sample, which has greatly expanded the ability to track T cell responses in any immune response⁷⁴. Using such methods to study human responses has been a necessity, but the fact that these natural infections and vaccine responses are so diverse calls into question the current practice in mouse immunology of monitoring single-cell MHC class I- or class II-restricted epitopes of ovalbumin in a given disease model. Also, just as with analysis of the repertoire of the B cell antigen receptor (BCR) response, the explosive growth and lower cost of DNA sequencing has greatly empowered the application of that technology to analysis of the TCR repertoire. Thus, there is widescale use of both sequencing of the TCRs of different populations of T cells and high-throughput single-cell methods. Particularly noteworthy in the application of bulk sequencing of TCRs of specific T cell populations is the demonstration that the initial T cell responses to specific pathogen types result in a broad array of CD4⁺ T cell functionality⁷⁵, but later analysis finds a much narrower, frequently monomorphic range⁷⁶. In single-cell analyses, substantial clonal expansion of most CD4⁺ T cells in a colon carcinoma tumor from a human patient has been shown, as well as a robust method for obtaining both α -chain and β -chain TCR sequences and phenotypic information⁷⁷. Single-cell RNA-seq methods have also been applied to T lymphocytes, but here the relatively small amount of mRNA in T cells has limited the ability of this method to derive TCR sequences, although some have reported success⁷⁸. For analysis strategies, while BCR sequences can often be grouped according to specificity by the observation of a cluster of somatic hypermutated immunoglobulin sequences, this mechanism does not exist in TCRs, so a major question becomes whether similar, but not identical, TCR sequences are really 'seeing' the same peptide-MHC ligand. In this context, computational methods hold the promise of being able to group TCR sequences in accordance to their specificities^{79,80} (Glanville, J. *et al.* personal communication), which would greatly aid the ability to interpret the data.

Overall, it seems likely that databases of BCRs and TCRs organized around the responses to specific pathogens will become an increasingly valuable resource with which to interpret adaptive immune responses. Questions about whether the diversity of the BCR or TCR response matters in terms of protection become very important in vaccine development, for example. Alternatively, is there a specific BCR or TCR specificity that is critical to protection? In the case of antibody

responses, this is clearly the case, as many antibody specificities are clearly not effective in inhibiting viral entry, for example. In T cell responses, it remains unknown whether a diverse response is the most critical factor or whether particular specificities are important. Thus, it is the hope that methods such as these will enlighten understanding not only of the evolution of the immune response within a host but also the features of protection at the molecular level.

Summary and future directions

While a good beginning has been made in the systems-immunology approach described here, much more is ahead, as immunologists have barely scratched the surface of many aspects. Also, there are major challenges in defining mechanistic and causal relationships. Right now research in this field is largely dependent on mice, but there will doubtless be situations for which inbred mice will not mimic a human pathway. In this context, humanized mice are one alternative but are still a work in progress. An alternative that should be developed more widely would be *in vitro* cell and organ systems that are entirely human and functional, which would allow mechanistic studies. Particularly important in this are the implications of systems immunology for medicine⁸¹, in which this approach could open many doors for the exploration of both human health and the myriad diseases that affect humans (Fig. 3). While it is known that many diseases involve inflammation, for example, this has not been explored with systems approaches in more than a few cases, so many specifics have yet to be discovered. Furthermore, while it is currently more difficult to discern biological meaning in the adaptive immune responses to an infection as compared to innate responses, the newer methods for analyzing the TCR or BCR repertoire are making it much easier to track this in people, so we think that there are many surprises in store here, such as a study showing the ability of lymphocyte physiology to be predictive of recovery following surgery^{82,83}. Currently, general medical practice and diagnosis do not make much use of immunology or appreciate how much it has advanced in the past decades. A case in point is that the main immunological metrics used widely in medicine are white blood cell counts and the complete blood cell count; the former was developed in 1915 and the latter was developed in 1959, it is time for an 'upgrade'!

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