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Systems Biological Analysis of Immune Response to Influenza Vaccination

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The last decade has witnessed tremendous progress in immunology and vaccinology, owing to several scientific and technological breakthroughs. Systems vaccinology is a field that has emerged at the forefront of vaccine research and development and provides a unique way to probe immune responses to vaccination in humans. The goals of systems vaccinology are to use systems-based approaches to define signatures that can be used to predict vaccine immunogenicity and efficacy and to delineate the molecular mechanisms driving protective immunity. The application of systems biological approaches in influenza vaccination studies has enabled the discovery of early signatures that predict immunogenicity to vaccination and yielded novel mechanistic insights about vaccine-induced immunity. Here we review the contributions of systems vaccinology to influenza vaccine development and critically examine the potential of systems vaccinology toward enabling the development of a universal influenza vaccine that provides robust and durable immunity against diverse influenza viruses.

INFLUENZA: WHAT ARE THE CHALLENGES?

Influenza carries a significant public health burden despite available therapeutic and preventative measures. Every year, seasonal influenza results in up to 1 million hospitalizations with 80,000 deaths in the United States alone (www.cdc.gov/flu/about/burden/index.html) and worldwide in up to 5 million severe cases with 650,000 deaths ([www.who.int/news-room/fact-sheets/detail/influenza-\(seasonal\)](http://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal))). In addition, antigenic shifts lead to widespread illnesses, as evidenced by five devastating pan-

demics in the past 100 years or so (1918, 1957, 1968, 1977, and 2009). Most recently, H5N1 and H7N9 viruses have emerged as potential pandemic threats causing lethal infections in humans, although they have not yet acquired the potential to spread from human to human (www.who.int/influenza/human_animal_interface/en).

Seasonal influenza vaccines are recommended for any individual 6 mo and older for the prevention of influenza illness. However, the effectiveness of these vaccines ranges between 10% and 60% (www.cdc.gov/flu/professionals/

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vaccination/effectiveness-studies.htm) as a result of several factors: (1) antigenic drifts caused by mutations in the influenza genome from selective pressure to evade preexisting immunity, which necessitates a selection of strain specific match on a yearly basis; (2) lack of durable protection; (3) poor cross reactivity against strains not included in the yearly vaccine formulation; and (4) suboptimal immunogenicity in certain populations such as patients at the extremes of age or with immunocompromising conditions. In addition, pandemic influenza vaccines, especially against avian influenza, are poorly immunogenic without the use of a high dose of hemagglutinin (HA) antigens (two doses each of 90 μ g of HA) or the addition of an adjuvant (most commonly oil-in-water emulsion adjuvants MF59 and AS03). Therefore, there is an urgent need to develop a universal influenza vaccine that could confer effective, durable, broad protection against influenza for all age groups. The development of a universal influenza vaccine is mostly hindered by the incomplete understanding of the mechanisms of vaccine protection. Historically, a hemagglutination inhibition (HAI) titer of ≥ 40 is the gold standard immunological parameter to predict protection from influenza illness after immunization. However, a HAI titer of 40 only represents a 50% protection rate against influenza viral infection at a population level (Hobson et al. 1972) and reflects just the antibody-mediated inhibition of viral attachment. Novel correlates should explore the non-neutralizing functions of antibodies (Jegaskanda et al. 2017) as well as the role of the cellular (Korenkov et al. 2018) and innate immune responses (Nakaya et al. 2011) to influenza vaccines. Despite recent breakthroughs in immunology, knowledge gaps still exist in our understanding of the complex interactions of the different components of the immune system to generate optimal immunity against influenza immunization.

SYSTEMS VACCINOLOGY

Goals and Proof-of-Concept Studies

Traditional immunological assays focus on serological correlates of protection, typically mea-

suring a limited number of immune parameters at a given time, making it difficult to capture the global architecture of the complex molecular processes and interactions involved in the immune response to influenza. In this context, the application of modern analytic tools of systems biology to vaccination opened a new era of exploration, one in which “systems vaccinology” could complement the traditional experimental methodology of reductionistic biology, by offering a broader, holistic viewpoint of the biological response to a vaccine (Pulendran et al. 2010).

Systems biological approaches, in fact, aim at capturing vaccination-induced changes happening at multiple levels and times by dissecting the cascade of biological events promoted by immunization. The technology uses high-dimensional, “omics” platforms (transcriptomics, proteomics, metabolomics, and lipidomics) that allow one to interrogate, in an unbiased and comprehensive manner, a plethora of biological responses at the mRNA, protein, metabolite, or lipid levels in an efficient way, thus minimizing costs and time, as well as the amount of biological specimen required. The large data sets generated via multiple high-throughput platforms can then be analyzed and integrated using computational and mathematical models in an effort to systematically explore and describe the physiological perturbations induced by vaccination as well as the intricate interactions between the different components of a biological system.

So far, systems biology has mostly studied successful vaccines as a way to probe the immune system to quantitatively and qualitatively assess their elicited protective immune responses (Pulendran 2014). The first examples of such systems studies aimed to understand and to predict the immune responses following immunization with the live-attenuated yellow fever virus vaccine YF-17D. By profiling the blood transcriptome of subjects vaccinated with YF-17D, two independent teams identified innate gene signatures of vaccination composed of type I interferon, inflammasome, and complement genes (Gaucher et al. 2008; Querec et al. 2009). In addition, Querec and colleagues detected early transcriptional changes that accurately predicted both the magnitude of CD8⁺ T cells and



the neutralizing antibody responses to YF-17D (Querec et al. 2009). Following these successful proof-of-concept studies, the field has applied systems biological tools to study vaccines against different pathogens (Li et al. 2014, 2017; Qi et al. 2016; Kazmin et al. 2017; van den Berg et al. 2017), with a special focus on seasonal influenza vaccines (Bucasas et al. 2011; Nakaya et al. 2011, 2015, 2016; Furman et al. 2013; Tsang et al. 2014; Hagan et al. 2019).

First Lessons from Systems Biology of Seasonal Influenza Vaccines

Influenza vaccines were first developed in 1938 in the form of virus-inactivated vaccines. Nowadays, many influenza vaccines are currently available. Most of them are inactivated and administered intramuscularly, trivalent or quadrivalent, and also available at high dose or adjuvanted for people 65 yr and older; others are optimized for intradermal delivery, whereas live attenuated influenza vaccines are given as a nasal spray (www.cdc.gov/flu/prevent/different-flu-vaccines.htm).

In one of the very first systems biology studies of influenza vaccination, we compared vaccine-induced responses of healthy young adults who received either inactivated influenza vaccine (IIV) intramuscularly or live attenuated influenza vaccine (LAIV) intranasally (Nakaya et al. 2011). Gene expression analysis, cellular immunophenotyping, and measurement of antibody titers have been at the core of the first systems-based influenza studies. Microarray analysis revealed very different responses between the two cohorts at the transcriptional level. LAIV recipients exhibited early signatures of differentially expressed genes, most of which are related to type I interferon (IFN) response (*STAT1*, *STAT2*, *TLR7*, *IRF3*, and *IRF7*), whereas the IIV group showed significant enrichment of genes highly expressed in antibody secreting cells (*IGH@*, *IGHE*, *IGHG3*, *IGHG1*, *IGHD*, and *TNFRSF17*). Strong innate immune responses, including early expression of IFN genes after vaccination, could accurately predict high versus low HAI responders to IIV 1 mo later, and these findings were also confirmed by an independent

team (Bucasas et al. 2011). In addition, we described strong vaccine-induced regulation of genes such as *TLR5*, *CASP1*, *PYCARD*, *NOD2*, and *NAIP*, suggesting new mechanistic links between host innate immunity and humoral responses to influenza vaccination. The importance of Toll-like receptor (TLR5) signaling for the generation of antibody responses against influenza is an extraordinary finding and will be commented on later in this section.

Given the importance of influenza vaccination in the elderly, the demographic group most affected by influenza, we subsequently applied systems biological approaches to compare the immune responses to influenza vaccine in young versus older adults across several influenza seasons (Nakaya et al. 2015). When compared to the younger group, the elderly exhibited diminished B-cell responses but increased frequency and activation of natural killer (NK) cell responses after vaccination, as well as an enhanced monocyte response pre- and postvaccination. Transcriptionally, we found a greater number of both up- and down-regulated genes observed in the younger group on day 1 after vaccination, which also extended to IFN-related genes.

Past systems biology studies have also shown the importance of baseline immune status in shaping immune responses to influenza vaccination. In a study from the Center for Human Immunology (CHI), Tsang et al. found that pre-vaccination cell populations, independent of subject's age, gender, and preexisting antibody titers, can accurately predict immune responses to influenza vaccine (Tsang et al. 2014). In addition to the CHI, the Human Immunology Project Consortium (HIPC) analyzed multiple cohorts of individuals vaccinated with influenza and found nine genes (*RAB24*, *GRB2*, *DPP3*, *ACTB*, *MVP*, *DPP7*, *ARPC4*, *PLEKHB2*, and *ARRB1*) and three gene modules at the baseline that were significantly associated with the magnitude of the antibody response postvaccination (IHIPC-CHI Signatures Project Team and HIPC-I Consortium). Based on our analysis of different influenza vaccine studies, we noted several B-cell- and T-cell-related modules whose expression before vaccination was positively correlated with an increased antibody re-

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sponse to vaccination. In contrast, modules related to monocytes were negatively correlated with antibody responses highlighting the concept that inflammatory responses at baseline might be detrimental to the induction of vaccine-induced antibody responses (Nakaya et al. 2015). In a separate experiment, Furman and colleagues had previously reported that they could distinguish between good and poor HAI responders by analyzing the prevaccination antibody repertoire against HA protein regions with established neutralizing properties (Furman et al. 2013). The baseline signatures identified by systems biology approaches can serve as predictive biomarkers that would allow for personalized interventions by identifying prior to vaccination potential responders versus nonresponders. Nonresponders could benefit from a tailored immunization regimen using modified formulations to improve immunogenicity (adjuvant, high dose of HA antigens), a boost mid-season to avoid waning immunity, or a modified route of vaccine administration (e.g., intradermal injection or through microneedle patch [Rouphael et al. 2017]) to better target the skin, an immunologically rich organ (Rouphael and Mulligan 2017).

Biology Taught by Systems Vaccinology: The TLR5 Story

Among the promises of systems vaccinology is the discovery of new biological mechanisms induced by vaccination and the generation of novel data-driven hypotheses that could further the field. In fact, hypotheses arising from systems studies can be mechanistically tested in follow-up experiments, and emerging results, in turn, promote new translational research with important implications for the clinic. The discovery of the crucial role of TLR5 signaling and gut microbes in shaping immunity to influenza vaccination is a perfect example of the full potential of systems vaccinology (Fig. 1).

In our first study of systems-level analysis of seasonal influenza vaccination, we noticed that the expression of the TLR5 gene on day 3 post-vaccination strongly correlated with antibody titers measured 4 wk later (Nakaya et al. 2011).

Because TLR5 is specific for the recognition of bacterial flagellin (Hayashi et al. 2001) and not associated with viral infections, we hypothesized that commensal microbes in the gut could be responsible for modulating immunity induced by influenza vaccination via TLR5. To test this, we designed an experiment in which TLR5-deficient mice, as well as mice treated with antibiotics (used to induce depletion of gut microbiota), were vaccinated with seasonal influenza and their vaccine-specific antibody responses compared with those of control mice (Oh et al. 2014). Our initial hypothesis was confirmed, as both TLR5-deficient and antibiotic-treated mice showed diminished antibody responses and lower frequencies of plasma cells compared to control animals. Interestingly, suboptimal antibody responses due to the lack of microbes or TLR5 deficiency could be improved by oral reconstitution with a flagellated (but not aflagellated) strain of *Escherichia coli*. Further, characterization of the gut microbiome of antibiotic-treated mice demonstrated that multiple bacterial communities, Gram-positive and Gram-negative, are involved in supporting humoral immunity to influenza vaccines.

These findings prompted us to ask if similar mechanisms occur in humans and to what extent the human gut microbiome regulates immunity to influenza vaccination. Therefore, to comprehensively assess the impact of the gut microbiota in clinical settings, we performed a study in which a cocktail of three antibiotics (neomycin, vancomycin, and metronidazole) was administered orally to healthy young adults for 5 consecutive days, from 3 d before to 1 d after vaccination with seasonal influenza (Hagan et al. 2019). We found that treatment with antibiotics resulted in a 10,000-fold reduction in the total number of gut bacteria and severely compromised bacterial diversity for more than 6 mo. As a consequence, the generation of IgG1, IgA, and neutralizing antibody titers specific to the H1N1 strain of influenza was affected, specifically in subjects with low preexisting antibody titers to influenza. Innate immunity was also altered, with enhanced frequencies of dendritic cells and early regulation of transcriptional modules associated with inflammation,

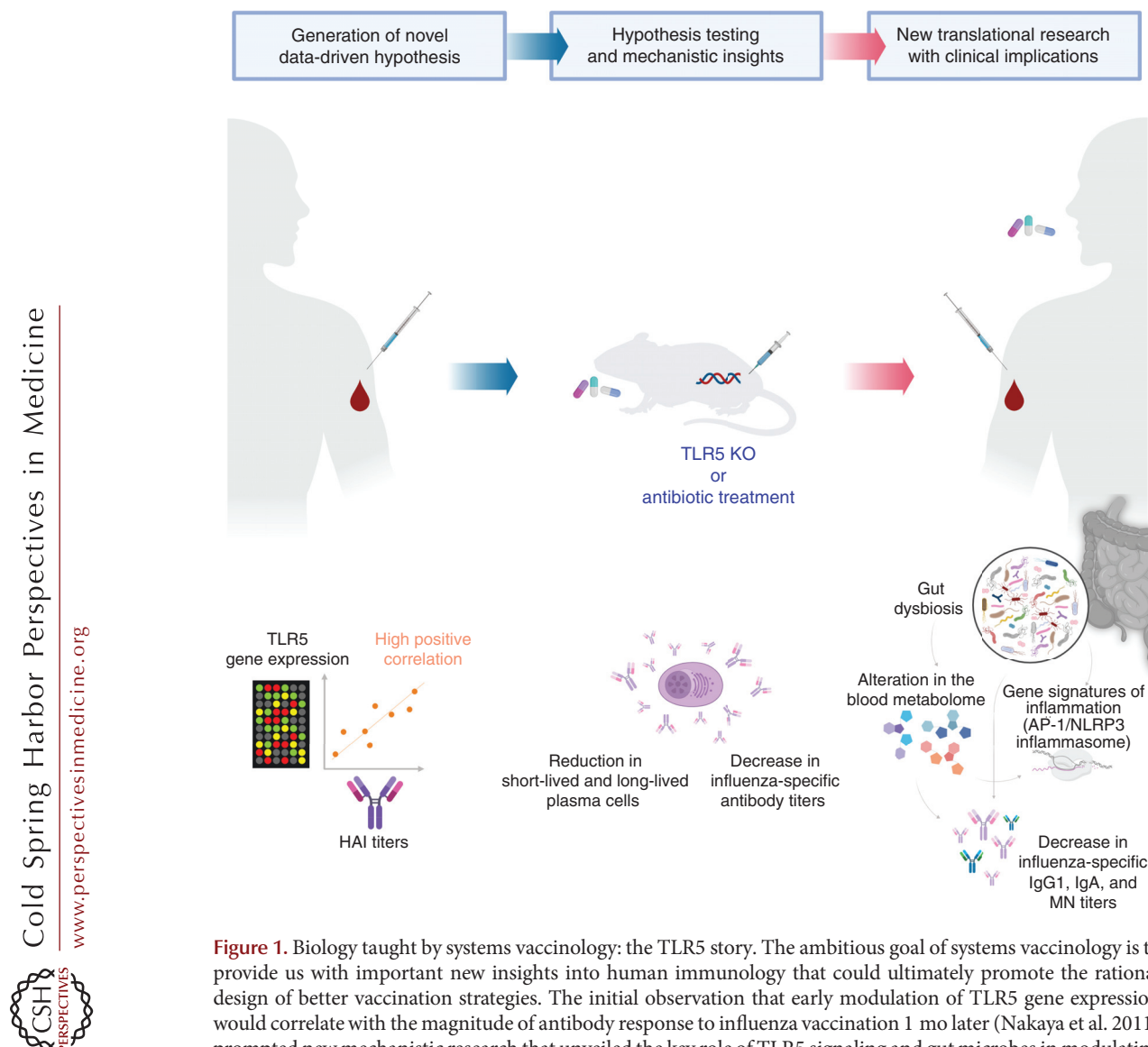


Figure 1. Biology taught by systems vaccinology: the TLR5 story. The ambitious goal of systems vaccinology is to provide us with important new insights into human immunology that could ultimately promote the rational design of better vaccination strategies. The initial observation that early modulation of TLR5 gene expression would correlate with the magnitude of antibody response to influenza vaccination 1 mo later (Nakaya et al. 2011) prompted new mechanistic research that unveiled the key role of TLR5 signaling and gut microbes in modulating immune responses to influenza (Oh et al. 2014). The results of this work inspired a clinical study that highlighted important biological interactions between antibiotics-driven depletion of the gut microbiome, inflammation, and alteration of blood metabolites, as well as the overall impact of gut dysbiosis on immunity to influenza vaccination in humans (Hagan et al. 2019). As antibiotics and vaccines represent globally used medical interventions, our findings provide important implications for clinical practice and public health.

including the transcription factor AP-1 and the NLRP3 inflammasome. Interestingly, we observed these same transcriptional changes in healthy elderly subjects immunized with the seasonal influenza vaccine in a previous study (Nakaya et al. 2015). These findings raise the

question of whether the process of “inflammaging,” low-grade chronic inflammation that develops with advanced age, may be associated with the alteration of the gut microbiome. Finally, up-regulation of genes related to inflammation strongly correlated with antibiotics-driven

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perturbation of the blood metabolome, particularly with a strong reduction in secondary bile acids (Hagan et al. 2019). By killing bacteria in the gut, antibiotics impair the conversion of primary bile acids (synthesized in the liver of the host) into secondary bile acids, whose production is instead under the control of gut microbes and is key to inhibit the activation of the NLRP3 inflammasome (Guo et al. 2016). Our results cast new light on how the microbiota can modulate secondary bile acid production and consequently inflammatory responses in humans, which can also affect immunity to vaccination.

HOW SYSTEMS BIOLOGY CAN HELP ADVANCE INFLUENZA VACCINE DEVELOPMENT

Identifying Signatures of Vaccine-Induced Immunogenicity and Protection

Unraveling the mechanisms of vaccine protection is of the utmost importance for providing new research directions that could catalyze the development of novel or improved vaccines. For this reason, systems biological approaches have been progressively incorporating additional immunological measurements and advanced computational tools in order to assess vaccine-induced immunity in the context of influenza more comprehensively.

Given the pivotal role of antibodies in providing protection against influenza, we expect humoral immunity to be examined in greater depth and from various angles by future systems' biological studies. Besides neutralization, emerging evidence in the field is supporting a significant role for nonneutralizing antibodies, which are not readily detectable by classical microneutralization (MN) or HAI assays, in mediating protection against influenza (Carragher et al. 2008; Henry Dunand et al. 2016; Asthagiri Arunkumar et al. 2019). "Systems serology" focuses on the ability of influenza-reactive antibodies with no evident neutralizing ability to engage innate immune cells through their Fc region. These antibodies are helpful for the mobilization of the powerful arsenal of effector functions typical of the innate immune system

(Boudreau and Alter 2019). Future work in this direction promises to shed new light on the totality of antibodies induced after influenza vaccination (and not only HAI or MN titers), thus adding "antibodyomics" (Ackerman et al. 2017) to the toolbox of cutting-edge technologies for the investigation of current and future vaccination strategies against influenza.

The development and implementation of untargeted high-resolution metabolomics into systems vaccinology studies have also recently added a new dimension of investigation to the field (Li et al. 2017; Hagan et al. 2019). In this light, we lately reported that the generation of H1N1-specific IgG1 responses induced following seasonal influenza vaccination are possibly dependent on metabolic pathways related to fatty acid metabolism, which is ultimately under the influence of the gut microbiome (Hagan et al. 2019).

Although antibodies represent a crucial line of defense against circulating virus particles, protection from influenza is likely to happen through multiple mechanisms, some of which may involve T cells, as shown for studies targeting the elderly population (McElhaney et al. 2006). These findings suggest that different components of the human immune system are at play against influenza, and their relative contribution may change with aging. In this context, clinical studies using T-cell-inducing, viral-vectored influenza vaccines have been proposed as a way to provide T-cell-based immunity against influenza in the elderly (Antrobus et al. 2012). Further, it is very well known that innate immune responses can significantly contribute to protection against influenza (Pulendran and Maddur 2015). The relatively new concept that innate immunity can also "remember" through epigenetic programming of myeloid lineage cells (Saeed et al. 2014; Netea et al. 2015), fueled by the rapid development of high-throughput methods to measure chromatin marks at the single-cell level (Cheung et al. 2018), opens a new avenue of research for the influenza field.

Moving forward, the emergence of current and future high-throughput technologies, followed by the powerful integration of multiple-omics data types using computational algo-

rithms such as the multiscale multifactorial response network (MMRN) (Li et al. 2017) will be key to reconstruct mechanisms of immunogenicity across orthogonal data sets and offer a broader picture of the biological processes underlying protective immunity against influenza.

Discovering Mechanism of Action of Adjuvants

Oil-in-water (o/w) emulsions have been established as strong adjuvants for inactivated influenza vaccines since the 1990s (Del Giudice et al. 2018). MF59 and AS03, the two most studied adjuvants in this category, are mainly composed of squalene, an intermediate lipid metabolite also produced by the human body as a precursor in the biosynthesis of cholesterol (Del Giudice and Rappuoli 2015). In addition to squalene, AS03 contains α -tocopherol, a type of vitamin E with immunostimulatory properties (Morel et al. 2011). Adjuvanted influenza vaccines are particularly beneficial for populations with sub-optimal immune responses, such as infants and the elderly (Della Cioppa et al. 2012; Wilkins et al. 2017).

After years of development, influenza vaccines containing MF59 and AS03 have been approved for use in different countries and are currently administered in hundreds of million doses of influenza vaccines worldwide. In the United States, a seasonal inactivated influenza vaccine formulated with MF59 (Fluad) has been licensed for persons aged 65 yr and older (www.cdc.gov/flu/highrisk/65over.htm), whereas, in Canada, the MF59-adjuvanted influenza vaccine has been recommended for use in the pediatric population (www.canada.ca/en/public-health/services/publications/vaccines-immunization/canadian-immunization-guide-statement-seasonal-influenza-vaccine-2019-2020.html). Both MF59- and AS03-adjuvanted H5N1 avian influenza vaccines have been stockpiled by the U.S. government in the event of a pandemic emergency (www.cdc.gov/flu/pandemic-resources/pdf/pan-flu-report-2017v2.pdf, Pandemic Influenza Plan, update 2017). Further, in the recent past, emulsions were used to boost immunogenicity of the 2009 influenza pandemic vac-

cine formulations in the European Union (Johansen et al. 2009).

Although safety profiles and beneficial effects on vaccine immunity of emulsion-adjuvanted influenza vaccines have been discussed extensively (De Gregorio et al. 2013; Di Pasquale et al. 2015; Tregoning et al. 2018), most of our understanding of the modes of action of o/w emulsions is limited and comes from mouse studies (Calabro et al. 2011; Morel et al. 2011; Caproni et al. 2012). To date, the exact molecular mechanisms involved in the adjuvanticity of these compounds in humans remain elusive. Delineating the mechanisms by which these powerful adjuvants improve vaccine immunogenicity would allow for a more rational design and development of next-generation adjuvants with similar safety profiles to currently licensed compounds but enhanced, or more targeted, immunomodulatory properties (O'Hagan et al. 2017).

Systems biological studies have recently started to interrogate immune responses to adjuvanted influenza vaccines in humans. We previously reported that a MF59-adjuvanted TIV enhanced both vaccine-specific HAI titers and frequency of multicytokine-producing CD4⁺ T cells in infants of <2 yr of age, while also inducing more homogenous and robust transcriptional responses in comparison to nonadjuvanted TIV (Nakaya et al. 2016). MF59 contributed to a strong and transient expression of a large number of IFN type I response genes 1 d after a booster vaccination that was positively associated with HAI responses. As for AS03, Sobolev and colleagues (2016) previously described early and robust transcriptional signatures of both myeloid and lymphoid activation after vaccination with an AS03-adjuvanted H1N1 monovalent influenza pandemic vaccine in healthy adults, thus suggesting involvement of innate-like lymphocytes in the early phases of vaccine response. In a separate study, Howard et al. (2017) used systems-based approaches to explore the immune response to a monovalent H5N1 vaccine given with or without AS03. The authors measured transcriptional changes in sorted cell populations and reported 80 “core AS03 responsive genes” shared between neutrophils, monocytes,

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and dendritic cells on day 1 following immunization.

Despite these first encouraging studies, further research is needed to reveal more about the modes of action of influenza vaccine adjuvants in humans, particularly in consideration of the importance of these substances in eliciting protective immune responses in crucial circumstances, such as influenza pandemics, and in special populations, namely infants, immunocompromised individuals, and the elderly. Given the presence of squalene, an important lipid intermediate, in both MF59 and AS03, we believe that an important contribution to the understanding of how emulsion adjuvants work will derive from the measurement of metabolic phenotypes in future systems vaccinology studies applied to adjuvanted influenza vaccines. o/w emulsions have been shown to induce intracellular accumulation of lipids *in vitro* after cellular uptake, and they may trigger immune responses partially through alterations in lipid metabolism (Kalvodova 2010). Of note, Givord et al. (2018) recently documented profound transcriptional changes linked to lipid metabolism in the draining lymph nodes of animals injected with AS03. The authors found that changes in lipid metabolism were responsible for endoplasmic reticulum stress in myeloid cells, which ultimately affected cytokine production and IL-6-driven differentiation of T follicular helper (T_{fh}) cells. Similar evidence in clinical studies is currently lacking.

Identifying Signatures of the Persistence of Antibody Responses

Lasting immunity is a desirable feature of any vaccine. In the case of influenza, although strain-specific HAI titers induced after vaccination have been shown to maintain protective levels for several months in different studies (Plant et al. 2017), there is a significant waning in neutralizing titers within 6 mo. However, there is a variability in the persistence of antibody titers, and the amount of antigen, use of adjuvants, type of influenza strains included in the vaccine formulation, or characteristics of the individuals such as age, sex, and immunization history could

all affect the durability of antibody responses specific to influenza (Plant et al. 2018).

Although multiple systems biology studies of influenza vaccination have generated interesting observations so far with regard to the transcriptional mechanisms associated with the magnitude of vaccine-induced peak antibody responses, the early molecular aspects of antibody persistence have not been addressed. This gap is partly due to a lack of consistent antibody measurements at late time points after immunization (e.g., between 1 mo and 1 yr postadministration), with most studies mainly focused on capturing magnitude and quality of vaccine-specific antibodies at the peak of their accumulation, usually within 1 mo from vaccination. Also, the difficulty in collecting human tissue samples in which key B-cell populations reside often limits investigation to peripheral blood. Long-lived plasma cells (LLPCs), which are responsible for the long-term maintenance of protective antibody responses for decades and populate the human bone marrow (Slifka et al. 1998; Halliley et al. 2015), are not found in blood and are therefore difficult to interrogate using systems biological tools.

In an effort to identify early transcriptional signatures within peripheral blood associated with durable antibody responses to influenza vaccination, we previously measured HAI titers 28 d (d28) and 6 mo (d180) after immunization in a group of healthy young subjects who received trivalent influenza vaccine (TIV) (Nakaya et al. 2015). We then used a linear regression-based approach to subtract the d28 effect on the d180 response and looked for transcriptional changes associated with the relative persistence of HAI titers that were independent of antibody concentration (magnitude) at the peak of response on d28 (Fig. 2). Interestingly, we found no correlation between early plasmablast activity and the D180/D28 residual, suggesting that factors other than the expansion of short-lived plasmablasts are responsible for maintaining a long-lasting antibody response. However, our approach led to the identification of a strong correlation between blood transcription modules (BTMs) related to cell movement and adhesion, including early regulation of the gene

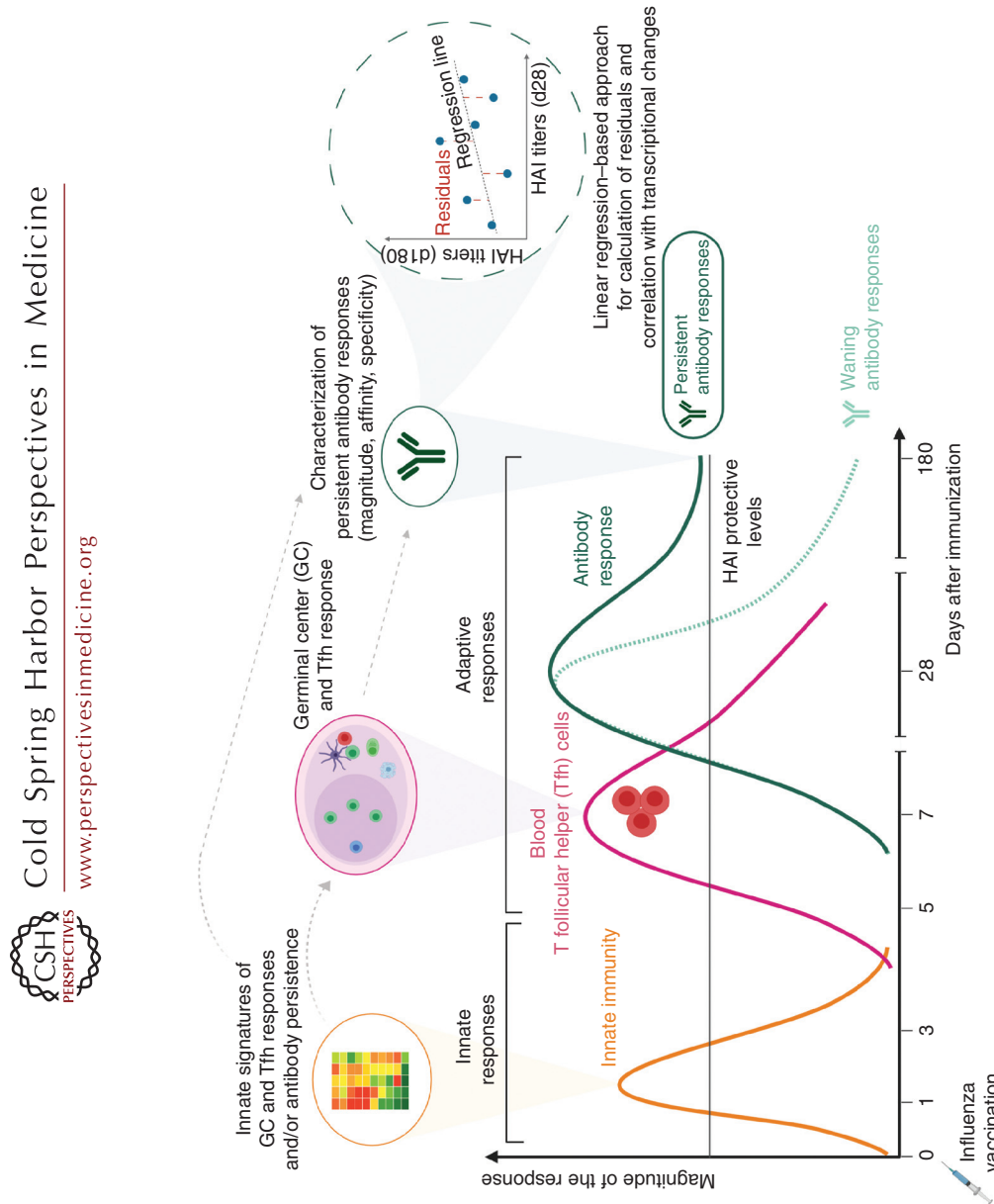


Figure 2. Identifying signatures of the persistence of antibody responses. The focus in influenza research is on the development of a universal vaccine that could provide long-lasting immunity against a broad spectrum of influenza viruses. A better understanding of the mechanisms that regulate the longevity of antibody responses after influenza vaccination is likely to facilitate this goal. We previously described a linear regression-based approach to identify early transcriptional changes (occurring 1–3 d postvaccination with seasonal influenza) specifically associated with the relative persistence of HAI titers 6 mo later (d180) (Nakaya et al. 2015). This analysis determined that up-regulation of SELP and other genes related to cell movement and adhesion could discriminate between persistent versus “nonpersistent” antibody responders 6 mo after vaccination. Given the importance of germinal center reactions in modulating the longevity of antibody responses, we believe that analysis of T_{fh} cells in the blood could be incorporated in this framework to gain new insights into the molecular mechanisms of antibody persistence.

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P-selectin (SELP), and antibody persistence. P-selectin, which plays an important role in adhesion and extravasation of leukocytes to the site of injury during inflammation (Wang et al. 2007), was more highly up-regulated on both days 3 and 7 following immunization in “persistent” antibody responders 6 mo after vaccination (Nakaya et al. 2015).

Further experiments are needed to validate these findings and assess the predictive ability of this antibody persistence signature across multiple influenza studies. With germinal center reactions being crucial for the generation of potent, long-lasting B-cell responses but often inaccessible in clinical settings, the measurement of surrogate markers such as antigen-specific antibody affinity or Tfh cells in the blood could be helpful to offer novel insights about the mechanisms of antibody persistence (Fig. 2; Hagan and Pulendran 2018). With this in mind, our laboratory is currently working on the identification of a transcriptional signature of antibody persistence following H5N1 (avian flu) vaccination with or without the AS03 adjuvant (M Cortese, T Hagan, S Khurana, et al., unpubl.). AS03 has been shown to induce long-lasting homologous H5N1-specific antibody response in humans (Chen et al. 2016), but the exact mechanisms involved remain unclear.

Defining Mechanisms and Correlates of Protection in Human Influenza Challenge Studies

The ultimate goal of systems biological studies applied to influenza research is to further our understanding of the mechanisms of immunogenicity and protection induced by vaccination and leverage this knowledge to develop novel or better vaccination strategies. Ideally, the best way of achieving this goal is to design clinical studies in which subjects who receive an influenza vaccine can be subsequently challenged with the pathogen. Such studies would provide us with a more direct measurement of vaccine efficacy and thus be extremely useful for the identification of biological markers that could discriminate protected from nonprotected vac-

cinees, therefore leading to the discovery of novel correlates of vaccine-induced protection.

Despite the fact that influenza challenge studies have been performed in humans for >80 yr (Sherman et al. 2019), an adverse outcome occurred in the early 2000s that drastically limited the adoption of human challenge models in influenza research worldwide for more than a decade (Ison et al. 2005). As a result, a major challenge has been the development and standardization of safe, reproducible, and ethically justifiable challenge models able to induce mild to moderate influenza disease (MMID) in a high percentage of unvaccinated participants without significant risks for the volunteers enrolled. However, there is renewed interest in the influenza human challenge models with several safe dose escalation trials with type A influenza strains circulated in recent years (Memoli et al. 2015; Han et al. 2019), thus bringing the application of systems-based tools to human challenges studies within reach.

So far, only a handful of studies have accessed high-throughput technologies to explore immunological or virological aspects of influenza challenge models in humans. Blood transcriptomic analyses of individuals exposed to either a H1N1 or H3N2 strain revealed distinct gene signatures postinfection for the two strains (Woods et al. 2013). Interestingly, the authors also identified an early transcriptional signature that could differentiate symptomatic from asymptomatic individuals well before the manifestation of symptoms (Woods et al. 2013). In a follow-up study using the same clinical cohort, Leonard and colleagues collected nasal washes and used next-generation sequencing (NGS) techniques to monitor genomic changes occurring to the influenza virus after infection (Sobel Leonard et al. 2016). Future studies should use systems-based approaches to identify mechanisms and correlates of vaccine-induced protection in the controlled human challenge models.

Antigen Discovery

The development of a universal influenza vaccines is a high-priority task for government health agencies and private organizations. The

National Institute of Allergy and Infectious Diseases (NIAID) recently unveiled a strategic plan for the pursuit of a universal vaccine suitable for all age groups that would protect against group I and II of influenza A viruses, possibly through multiple seasons (Erbelding et al. 2018).

Designing such a broadly protective vaccine would require a very different approach compared with currently developed seasonal influenza vaccines that mainly elicit antibody responses to the immunodominant globular head of the HA antigen. In fact, because the HA head domain undergoes fast antigenic changes and evolves continuously, most immune responses directed against this region are restricted to (almost) identical strains of influenza and are doomed to be nonuniversal in nature. To overcome this challenge, strategies seeking to develop broadly protective influenza vaccines have proposed to target more conserved components of the virus, such as the stem portion of HA (Neu et al. 2016). Given the immunosubdominance and steric shielding of the HA stem, efforts in this direction are focusing on immunization strategies that utilize either “headless” HA constructs or chimeric HA with a conserved stem but diverse HA heads (Graves et al. 1983; Krammer and Palese 2013; Krammer et al. 2013; Margine et al. 2013).

Significant help for a more rational design of a universal influenza vaccine comes from the development of high-throughput antibody sequencing platforms that allow the interrogation of the human B-cell repertoire with an unprecedented level of detail. This approach has the potential to guide epitope discovery and influenza vaccine design and development, similarly to what has occurred in the human immunodeficiency virus (HIV) field. The concept is simple: After natural infection (or vaccination), a number of subjects generate highly protective broadly neutralizing antibodies (bNAbs). Isolation of antigen-specific plasmablasts from these donors and subsequent single-cell antibody cloning techniques permit the rapid generation of thousands of monoclonal antibodies (mAbs) that can be further screened and characterized for their immunological functions and studied in complex with their target epitope via crystallog-

raphy or other methods for structural analysis. Identification of novel immunogenic epitopes and structural information derived from Ag-bNAb complexes can serve as a template for rational immunogen design and promise to expedite influenza vaccine development significantly. This methodology, previously described as “Reverse Vaccinology 2.0” (Rappuoli et al. 2016; Burton 2017), has led to the identification of extremely potent bNAbs against HIV (Doria-Rose and Connors 2009; Liao et al. 2013; Burton and Mascola 2015; Haynes 2015; Zhou et al. 2015) and influenza (Corti et al. 2011; Dreyfus et al. 2012; Lang et al. 2017; Nachbagauer et al. 2018; Stadlbauer et al. 2019). The main challenge for the field now is to leverage our understanding of the generation of bNAbs to design immunogens capable of inducing affinity maturation of B-cell clones responsible for the production of bNAbs (Rappuoli et al. 2016; Burton 2017).

THE LIMITS AND CHALLENGES OF SYSTEMS VACCINOLOGY

Although systems biology approaches show great promise in improving understanding of the molecular mechanisms of immunity to influenza infection and vaccine, there remain a number of current challenges that must be addressed. With the field mostly focusing on transcriptomic responses, a greater integration of new technologies, including metabolomics (Li et al. 2017; Hagan et al. 2019), proteomics (Lee et al. 2016), lipidomics (García-Sastre 2013), and glycomics, into systems biological approaches will enhance our ability to study antigen-induced perturbations at many different molecular levels.

Also, there is a need to reduce sources of biological and technical variability (Khadka et al. 2019). Standardization efforts will allow us to better integrate data obtained from different laboratories and different studies to enrich the knowledge gained from systems biology. Constraints on tissue collection are limiting the robustness of the approach with a focus on collecting and testing blood samples. Accessing a variety of other specimen types (e.g., lymph

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node aspirates, bone marrow aspirates, vaginal/rectal samples, and urine) would allow a deeper understanding of the immune responses.

In addition, by combining multiple data types and computational methods, systems biology approaches produce very rich data sets. It is of utmost importance for bioinformaticians to work closely with immunologists to convert data from high-throughput analyses to meaningful understanding of biological mechanisms of immune responses.

Ultimately, the key to improving these efforts will require proper integration and standardization of different-omics platforms with clinical, phenotypic, environmental, and microbiome data.

CONCLUSION

Given the dramatic advances in omics technologies, immune monitoring, bioinformatics, and machine learning, there is now an unprecedented opportunity to unravel the intricacies of the human immune response to influenza immunization and infection, ushering in a new era in vaccine and therapeutic development.

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Systems Biological Analysis of Immune Response to Influenza Vaccination

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