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Immunological assessment of influenza vaccines and immune correlates of protection

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Abstract

Influenza vaccines remain the primary public health tool in reducing the ever-present burden of influenza and its complications. In seeking more immunogenic, more effective and more broadly cross-protective influenza vaccines, the landscape of influenza vaccines is rapidly expanding, both in near-term advances and next-generation vaccine design. Although the first influenza vaccines were licensed over 60 years ago, the hemagglutination-inhibition antibody titer is currently the only universally accepted immune correlate of protection against influenza. However, hemagglutination-inhibition titers appear to be less effective at predicting protection in populations at high risk for severe influenza disease; older adults, young children and those with certain medical conditions. The lack of knowledge and validated methods to measure alternate immune markers of protection against influenza remain a substantial barrier to the development of more immunogenic, broadly cross-reactive and effective influenza vaccines. Here, the authors review the knowledge of immune effectors of protection against influenza and discuss assessment methods for a broader range of immunological parameters that could be considered in the evaluation of traditional or new-generation influenza vaccines.

Keywords

immune correlates; influenza virus; protection; vaccines

Today’s influenza vaccines, either inactivated influenza vaccine (IIV) or live-attenuated influenza vaccine (LAIV), are designed to elicit strain-specific neutralizing antibodies against hemagglutinin (HA), the major surface antigen of influenza viruses. Continual antigenic drift within the HA of seasonal viruses and occasional emergence of viruses with novel HA from animal reservoirs necessitates regular updating of influenza vaccine candidates. This is accomplished through the WHO Global Influenza Surveillance and Response System, which generates data for twice-yearly recommendations for the composition of seasonal influenza vaccines [1]. Unfortunately, even when influenza vaccines are well matched to circulating viruses, their effectiveness is generally lower in older adults, young children and those with certain medical conditions; the groups that are at higher risk

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of severe illness with influenza infection [2]. In seeking more immunogenic, more effective and more broadly cross-protective influenza vaccines for all age groups, the landscape of influenza vaccines is rapidly expanding, both in near-term advances and longer-term next-generation vaccine design. Recent advances for licensed inactivated vaccines include: the use of oil-in-water emulsion adjuvants for pandemic and seasonal IIV in some European countries and elsewhere [3]; virosomal vaccines in Europe [4]; high-dose seasonal IIV for older adults in the USA; intradermal seasonal IIV available throughout Europe, Australia and North America [5]; and the recent licensure of quadrivalent LAIV containing two influenza A and two influenza B vaccine viruses [6]. An influenza vaccine composed of full-length recombinant HA produced in insect cells was recently licensed by the US FDA [301], setting a precedent for this type of influenza vaccine production platform and laying the groundwork for future vaccines containing novel recombinant proteins. For the longer term, vaccines comprising novel antigen or adjuvant production platforms, DNA and vectored vaccines, together with vaccines that target conserved influenza A virus proteins or epitopes that elicit subtype cross-reactive responses, are all under development and clinical evaluation [7,8]. The latter ‘universal vaccine’ approach is particularly attractive for the pandemic situation because vaccines using well-matched HA-based approaches take several months to develop and produce [9]. The changing landscape in influenza vaccines and increasing challenges in conducting randomized placebo-controlled trials highlight the need for better surrogate immune markers as correlates of protection against influenza. Additionally, efforts to develop effective vaccines against emerging avian influenza threats are hampered by the inability to perform clinical efficacy or effectiveness studies and are in need of improved understanding and methods for standardized measurement of additional immune markers that correlate with protection. Such an immune marker would be statistically related with a protective outcome and be a predictor of vaccine efficacy, but may not be the causal immune effector(s) mediating protection [10]. The hemagglutination-inhibition (HI) antibody titer is currently the only universally accepted immune correlate of protection against influenza, even though it is recognized to be imperfect for assessing protection afforded by IIV among some age groups and inadequate for LAIV [11–13]. Improved and expanded immune correlates of protection against influenza are urgently needed to guide regulatory processes for pandemic and next-generation influenza vaccines. Here, the authors review the knowledge of immune effectors of protection against influenza and discuss assessment methods for a broader range of immunological parameters that could be considered in the evaluation of traditional or new-generation influenza vaccines. The authors focus on the methods that can be applied in both clinical and preclinical settings. Currently used assessment methods are depicted in FIGURE 1.

Immune correlates & immune effectors of protection against influenza

Control and clearance of influenza virus infection involves most components of the innate and adaptive immune system. Innate immune components keep the viral infection in check while the adaptive immune response, composed of T and B cells, develops with the aid of antigen-presenting cells (APCs) [14]. Influenza-specific antibodies, produced by activated B cells, play a major role in resistance to influenza infection, while antibodies to other viral proteins, in particular the neuraminidase (NA), also contributes, most likely to the

amelioration of clinical disease. In addition to directly limiting virus entry or release from infected cells, virus-specific antibodies may also aid in virus elimination through initiation of the complement cascade and antibody-dependent cellular cytotoxicity; mechanisms that have not been well examined with influenza.

Anti-HA serum antibody responses

Because of their ability to block virus attachment and entry into host cells, neutralizing antibodies directed against the globular head of the HA molecule are recognized to be the most powerful mediators of resistance to influenza infection and are considered the primary immune correlate of protection. The HI assay, because of its relative simplicity, has long been used as a surrogate assay for detection of virus-neutralizing antibodies in serum. Numerous studies have demonstrated resistance to influenza infection in persons with pre-exposure HI titers ≥32 or 40 [15–17]. This has led to the general convention of using a threshold HI titer of ≥40 as a measure of 50% reduction in the risk of influenza, sometimes referred to as seroprotective titer. Meta-analyses support the findings of individual studies but also demonstrate that higher serum HI titers are associated with higher rates of protection [18,19]. Although this HI titer threshold is the only universally accepted laboratory correlate of protection against influenza, several limitations of these earlier studies need to be considered in its generalized use for vaccine evaluation in all age groups. These include the use of attenuated challenge viruses to assess protection [17] and the fact that studies were generally conducted in younger adults that had acquired antibodies through natural infection rather than vaccination. In one study that did assess protection in adults following receipt of IIV, only serologic methods were used to detect influenza infection [16]. Recently, it has been recognized that serological end points, evaluated as a fourfold or greater rise in postinfluenza season HI antibody titer compared with preseason titer, may overestimate the protective effect of inactivated vaccines in adults because detection of such rises is compromised by already high preseason titers due to vaccination [20]. Indeed, a recent study in children that received an inactivated vaccine demonstrated that HI titers greater than 100 were associated with a 50% reduction in clinical influenza illness in laboratory-confirmed cases and HI titers over 200 predicted higher rates of resistance to clinical illness [21]. On the other hand, Ohmit *et al.* demonstrated that although absolute postvaccination HI antibody levels correlated with protection in adults vaccinated with either IIV or LAIV, IIV failures in particular had high HI titers that were not protective, indicating that perhaps quality as well as quantity of HI antibody, or indeed other immune parameters, need to be considered [11].

Recently, multiple groups have described the isolation of human monoclonal antibody (mAb) directed against the stem region of HA from memory B cells of adults exposed to influenza virus through infection or vaccination. These mAbs bind to non-contiguous epitopes within the highly conserved stem region of HA, blocking conformational changes in HA needed for membrane fusion, thus inhibiting virus replication [22–25]. These broadly cross-reactive antibodies exhibit neutralization activity *in vitro* and prophylactic and therapeutic protection *in vivo* against challenge with multiple virus subtypes, making them a target for broadly cross-reactive vaccines [26,27]. Such antibodies were also shown to contribute to clearance of virus-infected cells through antibody-dependent complement

cytotoxicity, further demonstrating that anti-HA antibodies may inhibit and/or reduce virus replication and disease impact by multiple discrete mechanisms [28].

Anti-NA serum antibody responses

NA, the second glycoprotein on the influenza virus surface, has sialidase activity that promotes virus release from infected cells [29]. Antibodies to NA inhibit release of the virus from infected cells, reduce virus replication and prevent disease in animal models and humans [30–34]. In humans immunized with inactivated vaccines bearing relevant human NA but irrelevant (equine) HA antigens, the level of serum anti-NA antibody was inversely related to the clinical illness observed. The highest anti-NA titers in these studies were associated with asymptomatic infection following homotypic experimental virus challenge or natural infection [32–35]. Clements *et al.* also found that anti-NA serum antibody acquired through natural infection or immunization with live-attenuated or inactivated vaccines was significantly associated with reduced virus replication in volunteers experimentally challenged with wild-type influenza A viruses [36]. Epidemiological studies suggest that anti-N2 NA anti-body induced by prior exposure to H2N2 viruses may have reduced the disease burden of the 1968 H3N2 pandemic [37].

Serum antibody responses to other viral proteins

In addition to the antibody responses generated against HA and NA, repeated exposure to influenza viruses elicits antibody responses to more highly conserved viral proteins; however, the range of conserved protein epitopes recognized and the role of such antibodies in promoting viral clearance or disease prevention in humans remain poorly understood. Studies in the mouse model provide some evidence as to the ability of antibodies to influenza A M2 protein and nucleoprotein (NP) to reduce virus replication and ameliorate disease. Although antibodies directed against the ectodomain of the M2 protein (M2e) do not neutralize the virus, numerous mouse studies have demonstrated the benefits of passive transfer of anti-M2e antibodies in reducing lung viral titers and ameliorating disease [38–40]. Antibodies that bind to M2, which is abundant on the surface of influenza A virus-infected cells, can mediate antibody-dependent cell-mediated cytolysis, which contributes to protection in mice [41]. In humans, influenza A virus infection appears to elicit only weak, transient M2 antibody responses [42,43], although mAbs derived from human memory B cells that recognize native M2 protein show similar properties to murine counterparts [44,45]. The relative conservation of the M2e sequence, particularly among human influenza viruses, makes it an attractive target for the development of more broadly cross-reactive influenza vaccines [7,46]. Early studies demonstrated that mAbs directed against the NP and matrix 1 (M1) proteins exhibited no protective effect in passive transfer experiments in mice [47]. However, more recently, polyclonal anti-NP antiserum derived from mice hyperimmunized with recombinant NP protein was shown to modestly reduce lung virus titers and lessen morbidity using a low-dose challenge model [48]. The use of whole-genome fragment phage display libraries to characterize the serum antibody response in influenza A H5N1-infected persons has identified antibodies with strong reactivity to the viral PB1F2 protein, a proapoptotic protein associated with virulence of influenza A viruses

in mice [49,50]. Whether anti-PB1-F2 antibodies are elicited by other influenza A subtypes and their role, if any, in protective immunity remain to be determined.

Antibody responses in the respiratory tract

While it has long been recognized that immunoglobulin in the respiratory mucosa contributes to control of influenza virus, the relative roles and importance of locally produced IgA versus plasma-derived IgG remains controversial, but likely depend on the site of action within the respiratory tract. Polymeric IgA (pIgA) is the primary mucosal antibody that protects mucosal surfaces together with pentameric IgM [51]. Mucosal IgA can neutralize influenza viruses inside secretory epithelial cells [52]. In young adults, protection from virus replication or illness was significantly correlated with HA-specific nasal wash IgA acquired through natural infection or vaccination with LAIV [36]. By contrast, nasal wash IgG derived from plasma by passive transudation was associated with resistance to influenza in individuals that had acquired antibody through parenteral receipt of inactivated vaccine [36,53]. These results illustrate a fundamental difference in immune correlates of protection between LAIV and IIV [54]. The mouse model has been used to better understand the ability of pIgA and IgG to control influenza virus infection. Studies in IgA-deficient mice have suggested that IgA is not essential for reduction of virus replication in the nasal cavity, although others have argued that enhanced secretory IgM may compensate for a lack of IgA both in mice and humans [51,55]. Mice impaired in the transepithelial transport of pIgA are impaired in their ability to reduce influenza virus titers in the nasal cavity [56]. In the absence of other influenza-virus specific responses, pIgA alone was shown to neutralize and eliminate the virus from the murine upper respiratory tract and prevent virus-induced damage to respiratory epithelium. However, in mice with only high levels of influenza-specific serum IgG, transudated plasma IgG can neutralize newly replicating virus in the murine lung [57,58].

T-cell responses

T-cell immunity requires antigens to be processed within cells and presented on their surface bound to MHC molecules, known as HLA in humans. CD4 T cells recognize exogenous antigens that have been internalized, processed and presented in the context of HLA class II, while CD8 T cells recognize endogenous antigens produced inside the cell, such as in the case of an infecting virus, that have been processed and presented in the context of HLA class I. Although less efficient, some APCs, primarily dendritic cell subsets, have the capacity to cross-present internalized antigens in the context of HLA class I to CD8 T cells [59]. This exemplifies one of the fundamental differences between the mechanisms of IIV and LAIV. IIVs stimulate antibody production and can be internalized by APCs to stimulate CD4 T cells, but are incapable of active replication in cells and therefore less effective at stimulating CD8 T cells. LAIVs are capable of limited replication in cells, and therefore more effective at stimulating CD8 T cells in addition to CD4 T cells and antibody production.

Antibodies are capable of binding and neutralizing live virions, making the development of vaccines that stimulate a strong antibody response attractive for influenza protection. A

strong, strain-specific antibody response has the potential to afford neutralization without infection of host cells. T cells can be activated without infection of host cells, albeit less effectively in the case of CD8 T cells; however, they can only eliminate viruses after cellular infection via lysis of infected cells or by inducing an antiviral state in infected cells. Virus-specific CD4 T cells are critical to the development of protective immunity, primarily by helping B cells and CD8 T cells by secreting cytokines to support the immune response. However, a subset of CD4 T cells has been shown to have a direct cytotoxic function [60–63]. Virus-specific CD8 T cells eliminate infected cells by releasing cytotoxic granules such as perforin and granzymes or by inducing apoptosis through Fas/Fas ligand interactions. CD8 T cells also support the immune response through cytokine production. While CD8 T cells have been shown to play a role in protection from influenza, these responses are likely subordinate to antibodies in vaccinated individuals when vaccines are well matched to the circulating strains. Unfortunately, vaccines are not always well matched to the circulating strain due to the amount of time required to produce the seasonal influenza vaccine and the speed at which influenza mutates. In contrast to antibody epitopes, T-cell epitopes are mainly derived from internal proteins that are more conserved between subtypes and are able to confer immunity to heterologous as well as homologous influenza viruses [64–68]. For this reason, there is now heightened interest in the ability of influenza vaccines to generate antigen-specific T-cell responses, especially in the context of vaccines designed to protect against pandemic influenza, a situation in which antibodies may not be well matched to the emerging strain [69–71].

To determine the role of T cells in protecting against influenza and identify potential cellular correlates of protection, a few experimental human infections have been conducted using live, unattenuated influenza viruses. In earlier studies, 63 volunteers were inoculated intranasally with A/Munich/1/79 virus [67]. In these experiments, both antibody and cytotoxic T cells correlated with protection. In subjects lacking neutralizing antibodies, the level of influenza-specific cytotoxic T lymphocytes (CTLs) correlated with viral clearance, but not susceptibility to infection. These initial studies did not distinguish CD4 and CD8 T cells.

More recently, human subjects experimentally infected with either H1N1 A/Brisbane/59/2007 or H3N2 A/Wisconsin/67/05 demonstrated that pre-existing influenza-specific T cells were associated with protection in subjects with no detectable neutralizing antibodies [72]. The magnitude of the peak CD4 responses in these subjects correlated with reduced viral shedding, illness duration and total symptom scores. Responding T cells in these studies primarily recognized antigens from the internal M1 protein and NP. The T cells in these trials were good producers of IFN-γ as determined by enzyme-linked immunosorbent spot (ELISpot) assay, and likely played an important part in the antiviral response. In addition to pre-existing influenza-specific CD8 T cells capable of cytotoxic killing, a subset of subjects in this study were demonstrated to exhibit influenza-specific CD4 T cells capable of direct cytotoxic activity pre-vaccination. Despite the demonstration of substantial CD8 T-cell responses, CD8 T cells were not well correlated with reduced shedding or severity of disease in these experiments, likely due to the high variability in CD8 responses between subjects and the small number of subjects in the study. Sampling time in this study was also likely to contribute to difficulties in the evaluation of CD8 T-cell

responses as a significant proportion of influenza-specific CD8 T cells were likely localized to the site of infection at 7 days postinfection.

Older adults are considered at high risk for influenza-associated complications due to dysregulation of the immune system, termed immunosenescence. While this topic is beyond the scope of this article, immunosenescence and challenges associated with influenza vaccination in older adults are more comprehensively reviewed by McElhaney [73] and Reber *et al.* [74]. Young children are also considered at high risk for influenza-associated complications, due in part to a naive and developing immune system. Bodewes *et al.* [75], PrabhuDas *et al.* [76] and Hodgins and Shewen [77] provide comprehensive reviews of childhood immune development and challenges associated with influenza vaccination in children. Experiments evaluating protection in these populations emphasize the importance of T-cell responses. Experiments performed in adults aged 65 years and older demonstrated that T-cell responses correlated with protection from influenza infection [78,79]. Experiments in young children (age 6–36 months), also demonstrated a protective correlation with the level of IFN-γ-producing cells induced by a LAIV vaccine [80]. In these experiments, conventional HI titers were shown to be poorer correlates of protection in these at-risk populations. These experiments highlight the need not only for the establishment of alternative correlates of protection, but also for confirmation in populations of more diverse age and health status.

HLA binding & T-cell immunity

As described earlier, T-cell responses require presentation of antigens in the context of HLA. HLA complexes are some of the most polymorphic genes in the human genome. Over 1000 allelic variants have been identified, each with its own unique peptide-binding properties, and thus its ability to stimulate a respective T-cell response [81–83]. This has presented a major hurdle for the study of T-cell immunity and indirectly, their use as a correlate of protection.

The understanding of CD8 T-cell responses has increased significantly with the development of HLA class I tetramers. HLA class I tetramers are four linked HLA class I molecules presenting a defined peptide antigen. This technology allows identification of antigen-specific CD8 T cells. However, the polymorphic nature of the *HLA* genes has resulted in most studies being limited to a handful of common alleles, *HLA-A2* being the most prominent due to its high frequency in western populations [84–86]. Despite this drawback, significant progress has been made in understanding CD8 T-cell immunity. By contrast, understanding of CD4 T-cell responses has lagged behind due to difficulties associated with the development of HLA class II tetramers. This is unfortunate considering the importance of CD4 T-cell responses in the development of protective immunity.

Mouse studies have shown that mice lacking functional CD4 T cells exhibit severely impaired immune responses to influenza and significantly shorter lived immune memory [87]. In humans, antigen binding to HLA class II, and thus presentation to CD4 T cells, is important for immune development. The HLA class II alleles *HLA-DRB1*03* and *DQA1*0201* have been associated with seronegative or low antibody responses to hepatitis

B and/or measles vaccines, while *DQA1*0104* and *DPA1*0202* alleles were associated with high antibody levels [88–93]. A small study examining individuals who failed to mount a neutralizing antibody response to influenza vaccination found an unusually high frequency of individuals expressing the *HLA-DRB1*0701* allele and an unusually low frequency expressing the *HLA-DQB1*0603* allele, suggesting potential genetic correlates with vaccine failure [94]. Further study in this area may provide useful information to improve vaccines and provide protection in a broader proportion of the population.

Methods & criteria to assess humoral immune responses to influenza vaccines

The ability to elicit HA strain-specific serum antibodies is often used as a primary end point of influenza vaccine immunogenicity. Multiple host characteristics influence the ability to mount antibody responses to influenza vaccines, the primary ones being age and history of prior exposure to the virus, as well as the health status of the individual. A number of different methods are available to measure antibodies to the HA and have been described in detail elsewhere [95]. The HI assay is the most widely used to measure strain-specific anti-HA antibodies because of its relative simplicity and correlation with protection. This assay and several others are described in brief below.

HI assay

The HI assay is a surrogate assay for detection of neutralizing antibodies that bind around the globular head of the HA molecule, inhibiting binding to terminal sialic acids on glycoproteins and glycolipids on cell membranes. The HI assay does not detect all neutralizing antibodies that bind to the HA molecule, including those that recognize the conserved stem region [22–25]. Antibodies to HA that bind or block the receptor-binding site inhibit hemagglutination of red blood cells (RBCs) in the HI assay at a fixed virus and RBC concentration [302]. The species of RBC used will affect assay sensitivity. In general, turkey, guinea pig or human type O RBCs are preferred for HI assays with contemporary human viruses, while horse RBCs are preferred for H5, H7 and other avian subtypes [96–102]. To improve sensitivity of detection of human postvaccination antibody to influenza B HA, ether-treated influenza B viruses should be used in the HI assay, although the use of ether-treated antigen may reduce assay specificity and compromise detection of strain-specific responses [103].

The postvaccination geometric mean antibody titer relative to the prevaccination titer is a key measure of influenza vaccine response. However, because many individuals are seropositive for seasonal influenza viruses, with detectable prevaccination titers, other measures are often used. These include the mean fold increase in titer (postvaccination divided by prevaccination titer) and seroresponse rate (proportion of individuals that have a fourfold or greater rise in titer from pre- to post-vaccination titer, where seronegative individuals achieve a titer of ≥40). While the proportion of individuals achieving an HI titer ≥40 (often termed the seroprotective rate) is also used, this may not reflect a true measure of the vaccine response in populations with pre-vaccination titers. Nevertheless, these criteria are applied by regulatory authorities within the EU and USA, requiring

age-specific minimum criteria for licensure of influenza vaccines [303,304]. In statistical analyses on vaccine immunogenicity, particularly in adults, the effects of prevaccination titers (and prior influenza vaccination history) should be considered [104]. The effects of increasing age should also be considered; these include overall decreased responses due to immunosenescence [73,74] and the skewing of antibody responses to previously encountered viruses of the same subtype, known as 'original antigenic sin' [105].

Single radial hemolysis

Single radial hemolysis (SRH) is another assay that detects antibodies to HA. SRH is based on the immunodiffusion of antibodies in agarose gel containing complement and influenza virus bound to RBC [106,107]. The end point titers are read as a lysis zone, resulting from complement-mediated lysis of the RBCs when antibody is present. Experience with SRH over several decades in Europe has provided evidence for a threshold SRH value associated with protection; an SRH area of 25 mm^2 or greater is considered a 50% protective titer [108,109]. This threshold titer, or a ≥50% increase in zone size in postvaccination compared with prevaccination sera, are criteria used by EU regulators to evaluate vaccine immunogenicity [303]. However, relatively few laboratories have the expertise to perform this assay, particularly for large studies [109–112]. The SRH assay may be somewhat more sensitive than the HI assay for the detection of postvaccination responses, particularly for influenza B viruses [113,114].

Virus neutralization

Virus neutralization (VN) is a highly sensitive and specific method for detecting antibodies that inhibit virus entry or otherwise block virus replication, and has the advantage of directly measuring functional VN [102,115,116]. In Europe, quantification of serum antibody responses by VN assay is required for approval of pandemic influenza vaccines [305].

For consistency and higher throughput, microneutralization (MN) assays using cultured Madin Darby canine kidney (MDCK) cells in 96-well microtiter plates is a widely used form of VN for detection of neutralizing antibodies elicited by vaccination. Typically, a standard amount of virus is added to serial dilutions of serum and following a reaction time, the mixture is subsequently added to MDCK cells. Longer incubation times (≥72 h) are required for the detection of virus by hemagglutination activity and/or cytopathic effect on cell monolayers. Longer incubation periods may yield less consistent results [117]. The ELISA has been used in MN assays to detect viral NP following overnight culture [118,119,302].

VN assays may better detect low antibody responses elicited by prepandemic vaccines targeting avian H5 and H7 subtypes [120,121]. However, a current limitation is the lack of an established protective titer for neutralizing serum antibodies. For this reason, HI titers remain a primary end point for immunogenicity and licensure of vaccines against avian subtypes. Like the HI assay, the postvaccination geometric mean antibody titer relative to the prevaccination titer, the mean fold increase in titer and seroresponse rate are common expressions of neutralizing antibody responses.

Pseudotype neutralization assays using retroviral vectors expressing influenza virus HA and NA are now widely used and may more readily detect broadly cross-reactive HA

stem region antibodies in serum which are not detected by neutralization assays using live influenza viruses [24,122]. As vaccine strategies targeting such broadly cross-reactive antibodies are developed, optimal assays to assess immunogenicity in preclinical and, eventually, clinical trials remain to be established.

Detection of NA inhibition antibodies

Recent improvement in detection methods for anti-NA antibodies has been stimulated by renewed interest in the role of these antibodies in protection against influenza, in part because of the recognition that anti-NA antibodies may elicit more broadly cross-reactive immunity against drifted virus strains or emerging influenza viruses [123,124]. Although currently licensed IIVs all contain NA, there is no regulatory requirement to quantify the amount of NA protein, which likely varies depending on the virus subtype and vaccine product [125]. For LAIV, NA antigen load presumably depends on the amount of replication the vaccine virus undergoes in the vaccinated host. Traditional methods to measure functional NA inhibition (NI) antibodies are based on the classical NA assay [126], which detects released sialic acid by the periodate–thiobarbituric acid reaction [302]. More recently, a miniaturized version of this original NI assay has been developed as a more practical approach for serologic evaluation of anti-NA antibodies induced by vaccination [127]. Additionally, an assay developed by Lambré *et al.* that uses peanut agglutinin lectin to detect galactose cleaved from sialic acid is gaining popularity as a method for higher-throughput detection [128,129]. A complication common to all NI assays is the need to exclude the effect of antiHA antibodies, which can sterically block NA enzymatic activity. Typically, this is achieved through the use of reassortant viruses with a relevant NA, but HA from a nonhuman subtype that is unlikely to cross-react (e.g., H6). Alternatively, use of purified NA protein or virus-like particles has been reported [129,130]. Unlike the HI assay, there are no standard approaches for the determination of anti-NA antibody responsiveness. In adult vaccinees with detectable prevaccination NI titers, a fourfold or greater rise in postvaccination titers has been used as a marker of response [131]. Recent studies using the lectin-based assay detected significant responses as a ≥twofold rise in postvaccination anti-NA antibody [129]. Moving forward, standardization of methods and criteria to detect NI antibody responses will aid in the development and assessment of new-generation recombinant vaccines that target NA as well as HA [128–130,132,133].

ELISA

ELISA is a useful method for detecting serum and nasal wash IgM, IgA and IgG class-specific responses to influenza vaccination, which can provide further information on the quality of the response [134–138]. Unlike the assays described above, which measure certain antibody functions, ELISA quantifies all antibody binding to the viral antigen, regardless of function. In a typical indirect ELISA, virus or recombinant HA is adsorbed to the wells of a microtiter plate, followed by sequential addition of serum sample, an enzyme-labeled antibody to detect bound immunoglobulin, and a substrate for colorimetric detection of binding. However, even when purified antigens are used, ELISA generally lacks strain and subtype specificity. The use of an ELISA-based method that assesses the ratio of antibody binding to native versus denatured antigen may better detect conformationally dependent neutralizing antibody and may better reflect protective antibodies [139]. A further

application of ELISA is that it can be modified to assess the avidity of serum antibody binding or detect nasal wash immunoglobulins, particularly IgA [140]. In previously seronegative recipients of LAIV, resistance to experimental influenza A virus challenge has been correlated with detection of nasal wash IgA by an HA-specific indirect ELISA [36,135]. However, there is no standardized method or reagents for the detection of local IgA antibodies. Use of a kinetic ELISA may decrease nonspecific reactions for the detection of nasal IgA responses [141]. Normalization of titers based on total IgA content of sample is necessary. Detection of antibodies above a threshold optical density value or detection of a ≥fourfold rise in normalized postvaccination titer were criteria of an influenza-virus specific response to vaccination in children that received trivalent LAIV [142]. Detection of ≥fourfold rises in serum IgA by indirect ELISA has been reported to be a more sensitive measure of response to avian LAIV than HI or VN titer measurement [143]. However, it is not known whether serum IgA responses are in any way relevant for protection [144].

Detection of anti-M2e antibodies

Influenza A virus M2 is a target for novel vaccines and therapeutic antibodies because of its genetic and functional conservation across influenza A subtypes [45,145]. Evaluation of M2 antibody responses in preclinical M2 vaccine studies has typically been achieved through the use of short synthetic M2e peptides as antigens in conjunction with an indirect ELISA assay format [43,146–149]. This approach, although relatively straightforward, may not detect antibodies directed against conformational M2 epitopes displayed on the native tetrameric form of the M2 molecule, which in preclinical studies correlated well with the protective efficacy of an M2-based vaccine regimen [150]. To overcome the possible limitation of synthetic peptide ELISAs, several groups have developed cell-based ELISAs or flow cytometry-based approaches to detect antibodies using cell lines expressing native forms of M2 [43,151]. Assays that detect antibodies recognizing native M2 protein may be preferred for the evaluation of M2-based vaccines in clinical trials. Such assays are sufficiently sensitive to detect modest rises (two- to four-fold) in antibodies in a proportion of adults recently infected with seasonal influenza [43]. Additionally, several studies have demonstrated that anti-M2 IgG-mediated immune protection in mice was dependent on Fc receptors and NK cells or alveolar macrophages as the effector cell involved in antibody-dependent cellular cytotoxicity or cell-mediated phagocytosis [41,152]. Quantitative assays that measure killing of M2-expressing target cells may be a suitable means with which to establish a correlate of protection.

Methods to assess cell-mediated immune responses to influenza vaccines

Although it is well accepted that cellular immunity plays a significant part in immunological protection from influenza, there are currently no well-defined, broadly accepted cellular correlates of protection. However, there are a wide variety of techniques that have been developed and are frequently utilized for the quantitation of cell-mediated immune responses to influenza infection and vaccination. Any future cellular immune correlate of influenza protection is likely to have its basis in one of the following techniques.

ELISA & ELISpot assays

One of the most commonly measured immunological indicators is cytokine production. Cytokine production can be quantified in serum, alveolar lavage samples or culture supernatant using cytokine-specific ELISAs [153,154]. While ELISAs accurately measure the amount and type of cytokine produced, they are often less useful in clinical studies as they are unable to measure the type or number of cells producing the cytokine. When used to quantify cytokine production, ELISAs are usually used in conjunction with ELISpot assays to determine the number of cells producing a given cytokine or in conjunction with flow cytometry. In a common T-cell ELISpot assay, cells are plated at multiple densities into microtiter plates coated with a capture antibody specific for the cytokine of interest. Upon stimulation with antigen, the cytokine of interest is captured in a zone around the producing cell. This zone is visualized using antibodies conjugated to a reporter system that produces a precipitating substrate. Each spot corresponds to an actively secreting cell. These spots can be counted manually or through the use of an automated reader. In addition to measurement of cytokines, ELISpot assays have also been utilized to measure other functional proteins, such as granzyme B production by cytotoxic T cells [155–162].

Although they are central to production of protective antibodies, B cells are less commonly assessed in influenza research aside from their indirect measurement through antibody titers. When B cells are assessed directly, they are primarily measured using B-cell ELISpot, which utilizes microtiter plates coated with the antigen of interest to quantitate antigen-specific B cells. This can be useful to assess the number of memory B cells with the potential for antibody production in an infection [70,163]. Flow cytometry is also often used for assessment of B cells; however, it does not accurately define antigen-specific B cells, and antigen specificity must often be inferred from the expansion of B-cell populations at time points after vaccination or infection. B-cell flow cytometry is often coupled with B-cell ELISpot and/or antibody ELISAs as analysis by flow cytometry often gives other useful information such as B-cell condition and/or activation status. The advantages of ELISA and ELISpot assays are that they are relatively inexpensive in comparison to other cellular immune assays, and are amenable to high-throughput analysis.

Cellular proliferation assays

Cellular proliferation assays take advantage of the fact that adaptive immune cells rarely proliferate, except upon antigenic stimulation. The earliest form of this assay uses incorporation of a radioactive DNA nucleotide, ^{3}H-thymidine, into newly synthesized DNA in dividing cells after stimulation with antigen [153,155,156,164,165]. Cells are then lysed and collected onto glass-fiber filter paper. The amount of radioactive material collected on the filter paper reflects the proportion of dividing cells. Results are reported as a stimulation index, typically defined as the counts per minute (cpm) of stimulated cells divided by the cpm of unstimulated cells. A limitation of this assay is that it does not distinguish the types of responding cells, nor directly measures the number or percentage of dividing cells. Dividing cells are a mixture of CD4 and CD8 T cells and B cells, and are measured indirectly as a function of the magnitude of the radioactive signal. Although ^{3}H-thymidine proliferation assays are still utilized, they have largely been replaced with nonradioactive assays with the flexibility of assessing cell phenotype and direct

quantification of dividing cells. One method commonly used is similar to ^{3}H-thymidine incorporation, but replaces thymidine with 5-bromo-2′-deoxyuridine (BrdU). Incorporated BrdU is detected using a specific mAb conjugated to a reporter compound. The reporter compound can be varied to give this assay system significant flexibility. Conjugation of enzymatic compounds such as horseradish peroxidase or alkaline phosphatase can be used for a quantitative colorimetric or chemiluminescent readout similar to ELISA [157]. Conjugation with a fluorescent compound allows application to flow cytometry (discussed below) for phenotyping of dividing cells [166–168]. Another common method for assessment of cellular proliferation is by 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) staining. Cells stained with CFSE are stimulated with the antigen of interest. Proliferating cells show a decrease in CFSE fluorescence intensity by flow cytometry (discussed below), which allows for assessment of cellular phenotype and function of dividing cells [84,158,166,169–171]. Proliferation assays are often used as a means of characterizing the adaptive immune response. However, they can also play an important role in epitope mapping and determinations of immunodominance to both vaccination and infection. These assays use an array of stimulating peptides generated from the virus or vaccine of interest with cellular proliferation as the readout.

Cytotoxicity assays

The need for quantifying specific cellular immune function has spurred development of a variety of functional assays including cytotoxicity assays, cytokine ELISAs and ELISpot assays. Cytotoxicity assays quantitate the ability of cytotoxic T cells and/ or NK cells to lyse virally infected cells. These cellular functions are likely important for viral clearance. Cytotoxicity has traditionally been measured by release of chromium-51 (^{51}Cr) from lysed ^{51}Cr-labeled target cells [157,159,169,172–174]. Drawbacks to using this assay are primarily associated with the use of radioactivity. An alternative to ^{51}Cr uses the release of lactate dehydrogenase (LDH) from lysed cells [154,175,176]. The released LDH converts lactate to pyruvate, which in turn reacts with tetrazolium salt resulting in a quantifiable color change. In both the ^{51}Cr and LDH release assays, cytotoxicity is measured across multiple effector:target ratios. Cellular cytotoxicity is reported as the percent specific lysis defined as: (experimental lysis – spontaneous lysis)/(maximum lysis – spontaneous lysis) × 100.

CTL assays, such as fluorolysometric (FL)-CTL and fluorescent antigen-transfected target cell (FATT)-CTL assays, have been developed which directly measure elimination of fluorescently labeled targets by cytotoxic effector cells [85,172–174,177]. Cytotoxicity assays have also been developed that quantify target cell killing by CTL-induced apoptosis through the measurement of apoptotic mediators such as caspase 3 [160]. All current cytotoxicity assays measure the elimination of target cells, but are unable to directly measure the number of cytotoxic effector cells. However, a method developed for the ^{51}Cr-release assay using limiting dilution of effector cells can take advantage of statistical analysis to estimate the number of cytotoxic effector cells [63,86,178,179]. This method is likely applicable to the other types of cytotoxicity assays mentioned above.

Flow cytometry

Flow cytometry is quickly becoming the predominant method of cellular immune assessment. Flow cytometry uses fluorescently labeled monoclonal antibodies to simultaneously measure multiple phenotypic and/or functional markers. The commercial availability of a vast array of monoclonal antibodies makes this technique extremely versatile, with the ability to tailor measurements to the particular needs of the study. The response of multiple cell types can be assessed in a single sample along with multiple functional indicators such as intracellular cytokine or cytotoxic granule production, activation state, or in conjunction with BrdU or CFSE for cellular proliferation [70,71,84–86,157–163,166,169–171,173,174,180,181]. MHC tetramer staining can be performed to identify antigen-specific T cells by flow cytometry, but is performed primarily in mouse models [154,166–168,171] due to extensive genetic polymorphism of human HLA (discussed earlier). When performed in humans, tetramer staining is often limited to a subset of samples [85,155,157,160,169,172]. While the versatility of flow cytometry is a significant advantage, its complexity compared with many of the other techniques requires highly skilled labor to be used effectively [182,183]. Additionally, its higher cost relative to other techniques often limits its use to a subset of samples in large clinical trials [182]. High-throughput applications have been developed for this technique, easing some of the labor intensity associated with flow cytometry.

Cell-mediated correlates of protection for influenza will be defined by well-designed clinical efficacy or effectiveness studies. Flow cytometry and ELISpot assays are likely the best candidates for identifying these correlates. The versatility of these two assays allows assessment of an extensive breadth of cell-mediated immuno logical function. Both of these techniques are also amenable to high-throughput analysis. A concerted effort for the standardization of these techniques is needed if cellular correlates of protection are to be established for influenza.

Standardization of assays

Interlaboratory variability in assay techniques and determination of assay end points poses a considerable challenge for comparing the immunogenicity of influenza vaccines in clinical trials. While there are currently accepted criteria for HI and SRH assays, many of the serological and cellular assays lack such established criteria. Lack of standardized protocols and knowledge as to what constitutes a positive response to vaccination and how it relates to protection leads to variability in results between laboratories. Some laboratories utilize fold increases in pre- to post-vaccination titers for some serological assays, using criteria similar to the HI assay, while others rely on statistical significance to determine immunogenicity. T-cell assays in particular are prone to these concerns. A wide variety of assay protocols are utilized by a number of laboratories. Statistical significance is usually used as the determining criteria for immunogenicity without an understanding of how the responses relate to protection from disease. A concerted effort by stakeholders is needed to establish standardized techniques and develop the knowledge base needed for establishment of assay criteria.

Recent global interest in development of H5N1 prepandemic vaccines and the 2009 H1N1 pandemic has renewed interest to evaluate the extent of interlaboratory variability and develop tools for improved standardization to aid the regulatory process. International studies that have compared both inter- and intra-laboratory variation among HI and VN assays have demonstrated poor reproducibility of these assays, with the more technically demanding VN assay showing even greater interlaboratory variability than the HI assay [117,184,185]. For the HI assay, the species and method of standardization of RBC as well as efficiency with which sera are treated to remove nonspecific inhibitors may contribute to variability in results between laboratories. For VN assays, differences in protocols, quality of cells used and, in particular, the amount and standardization of virus used, likely contribute to poor reproducibility and interlaboratory variability. The development and use of a standard antibody reagent to normalize results within a laboratory reduces interlaboratory variability in HI and VN assays by at least 50% [184–186]. Recently, the EMA assessed serologic assay variability between vaccine manufacturers and European regulatory agency laboratories using a defined subset of sera from A(H1N1)pdm09 clinical trials [187]. The substantial variability in antibody titers observed among manufacturer and regulatory laboratories was greatly reduced when absolute titers were calibrated relative to the A(H1N1)pdm09 international standard. Therefore, the use of antibody international standards is a new and powerful approach to improve inter laboratory agreement for serologic assessment of influenza vaccines. The use of standard operating procedures to qualify serologic assays should also improve intra- and interlaboratory variability [188]. The newly formed Consortium for the Standardization of Influenza Seroepidemiology is currently investigating options for the improved harmonization of influenza serological assays [189].

Expert commentary & five-year view

The conduct of well-designed clinical efficacy or effectiveness studies in different age groups with defined immunological endpoints is key to the development of new and reliable immune correlates of protection against influenza illness. Although the HI titer of ≥40 remains a useful and important benchmark for the qualitative assessment of protection in a population, there are limitations for the generalized use of this long-held correlate for assessing IIV-induced protective immunity in populations of different ages and risk groups [11,21,78,79]. New influenza vaccine strategies may target specific segments of the population, and it will be important to develop immune correlates that are similarly age group specific. The lack of knowledge and qualified methods to measure alternate immune markers of protection against influenza remain a substantial barrier to the development of more immunogenic, broadly cross-reactive and effective influenza vaccines. The lack of any laboratory correlate of protection for LAIV is problematic for future licensure of this technology, which is an important component of the WHO Global Influenza Action Plan to build global pandemic vaccine capacity in developing countries [190].

There is a growing consensus among influenza experts and regulatory authorities that qualified, well-standardized assays that quantify non-HI anti-HA antibodies, antibody directed against other viral components (e.g., NA or M2) or T-cell responses are needed to establish additional immune correlates of protection to support licensure of next-generation

influenza vaccines [191–193,306]. TABLE 1 outlines prospective correlates of protection for influenza and assays currently used for assessment. European regulatory agencies are currently reassessing guidelines for influenza vaccine licensure to include vaccine type and subpopulation-specific considerations as well as a broader range of criteria for immunogenicity [307].

Since the relevance of VN assays is now well recognized, there is an urgent need to incorporate these assays into vaccine efficacy studies to identify a correlate of protection against laboratory-confirmed seasonal influenza. This should also aid in regulatory evaluation for vaccines against pandemic threats. Furthermore, additional attention to the development of standardized methods to quantify stem region-specific subtype cross-reactive antibodies, and/or those that mediate cell- or complement-mediated cytotoxicity is warranted. For stem-region antibodies, assays that detect inhibition of cell–cell fusion or proteolytic activation (trypsin cleavage) of HA have been used to understand the neutralizing mechanism of these mAbs [22,24]. The development of higher throughput assays that quantify fusion inhibition in HA-transfected cells may be one means to measure functional stem-region antibodies in human sera. Although assays to measure cross-reactive antibody-dependent cell or complement-mediated cytotoxicity activity on virus-infected cell lines have been described [28,194], further development of standardized methods is needed. For cell cytotoxicity assays, there is a need to standardize both the target and effector cells. This could be achieved by the use of a continuous cell line expressing the desired viral target (e.g., HA, NA or M2) and a human cloned effector cell (e.g., NK cell); appropriate human positive and negative control antibodies are also needed. Recently, Jegaskanda *et al.* described a novel antibody-dependent cell-mediated cytotoxicity assay that measured intracellular IFN-γ and degranulation (CD107a) expression by NK cells after incubation with immobilized influenza antigen and non-neutralizing antibody complexes [195]. Although this approach was evaluated using whole virus or purified HA, the use of alternate purified viral proteins may broaden the applicability of such an assay.

Substantial progress has been made in developing antibody standards for serologic assessment of pandemic and prepandemic vaccines, thereby establishing a process to reduce interlaboratory variability and promote comparability of different vaccines. This approach has not yet been adopted for seasonal influenza vaccines, but should be explored. Efforts are underway to unify and standardize methods for detection of NI antibodies, which may be a first critical step towards developing a laboratory-defined correlate of clinical protection. This may be particularly challenging for immune parameters such as anti-NA that may reduce disease severity rather than prevent infection and may require more intensive and costly studies to detect milder illness or asymptomatic infection. The human experimental challenge model may be a more controlled environment for this purpose, and has recently been used to evaluate a vector-based vaccine strategy [196].

Two areas of assay development warrant particular attention. Mucosal antibody responses likely contribute an important component of protection that is poorly quantified by existing assays, but could be particularly beneficial in describing correlates of protection for LAIV, and potentially some next-generation vaccines. Future LAIV trials should consider standardized sample collection and ELISA procedures for evaluation of nasal

immuno globulin and cross validation of methods by a central expert laboratory. Although quantification of cell-mediated immune responses to influenza vaccines has become more common, multiple methods are in use to assess vaccine immunogenicity. Of these, it is likely that for T-cell responses the IFN-γ T-cell ELISpot coupled with detection of $CD4^+$ and $CD8^+$ T-cell phenotypes by flow cytometry following influenza virus-specific stimulation of peripheral blood mononuclear cells may be the most amenable to standardization in method, reagents and response criteria. While some progress has been made toward validation of T-cell assays, more work is needed, ideally by an international consortium of laboratories that can identify optimal methods, reagents and criteria for assessment of T-cell responses such as has been established for HIV vaccine development [197,198].

In the short term, those supporting and conducting clinical studies, particularly those assessing efficacy or effectiveness, should consider incorporating a broader range of laboratory assessments to identify additional immune markers that may predict protection from laboratory-confirmed influenza illness or disease. Recognized challenges in this regard include the altered timing and larger volume of blood required for optimal detection of cell-mediated immunity compared with serum antibodies. In the longer term, new high-throughput global gene expression technology will allow for expanded analyses of the immune system networks that may identify immunological signatures associated with protective responses. This approach has already revealed fundamental differences in signatures elicited by IIV versus LAIV, and/or identified early molecular signatures correlated with subsequent anti-HA antibody responses [199,200]. In the future, this systems biology approach should have the power to define a complex of immune correlates of protection against influenza that are age group and vaccine specific.

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Key issues

- The hemagglutination-inhibition antibody titer is currently the major immune marker correlated with protection from influenza. However, this correlate may be less effective at predicting protection in populations at high risk for severe influenza disease and for the assessment of nontraditional influenza vaccines.
- Validated, well-standardized assays that quantify responses to non-hemagglutination-inhibition anti-hemagglutinin antibodies, antibodies directed against other viral components (e.g., neuraminidase or M2) or T-cell responses are needed to establish additional correlates of protection to support future licensure of novel influenza vaccines.
- A variety of immunological assays are currently utilized to assess the immunological response to influenza and influenza vaccines. Future correlates of immune protection will likely be derived from current, commonly assessed immunological functions measured by these assays in response to influenza infection and vaccination.
- There is the need for a concerted effort by institutions and industries currently involved in influenza research and vaccine development to use qualified and standardized methods to identify alternate immune markers as correlates of protection against influenza.

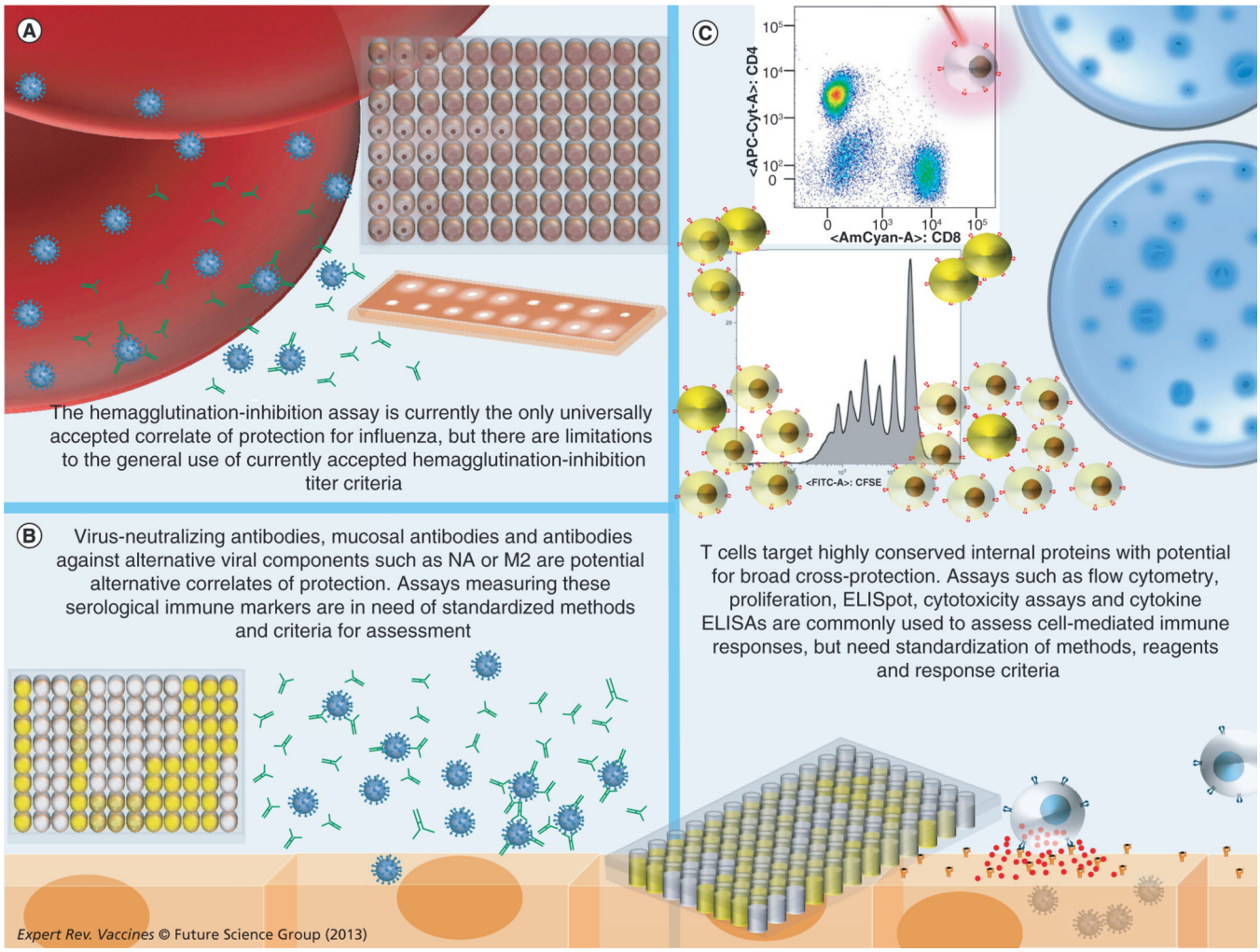

Figure 1. Assessment of immune responses to influenza.

(A) Antibody titers measured by the hemagglutination-inhibition assay are the gold-standard immune marker correlated with protection against influenza. However, there are limitations to the generalizability of the currently accepted hemagglutination-inhibition titer criteria, particularly in populations at high risk for severe influenza disease, and for the assessment of nontraditional influenza vaccines. Single radial hemolysis is also a recognized correlate of protection for influenza and is used by the EU for vaccine licensure. **(B)** A variety of serological assays are currently used to assess antibodies; some of these have the potential to identify alternative correlates of protection for both traditional and nontraditional influenza vaccines. Virus neutralization assays are becoming more widely used, but standardized methods and criteria for evaluation of titers associated with protection are lacking. Standardized assays that detect responses to M2 and neuraminidase are also needed. **(C)** T cells target viral epitopes primarily derived from internal proteins that have been shown to be highly conserved across viral strains. While assays assessing cell-mediated immune responses to influenza vaccination are becoming more commonplace, identification of key,

qualified assays are needed to develop cellular immune markers that may correlate with protection against influenza. ELISpot: Enzyme-linked immunosorbent spot.

Table 1.

Prospective correlates of protection for influenza and assays currently used for assessment.

Immune marker	Assay	Description
Serum anti-hemagglutinin antibody	Hemagglutination inhibition	Relatively simple, functional surrogate assay for the detection of neutralizing antibodies. Titer ≥40 recognized as immune correlate of protection and is used by international regulatory agencies for vaccine licensure purposes
	Virus neutralization	More complex than hemagglutination inhibition assay, but directly measures neutralizing antibodies and may be more sensitive to detect responses in some cases. No established titer that is associated with protection
	Single radial hemolysis	Directly measures functional antibody but not widely used. Zone of hemolysis of ≥25 mm2 is correlated with protection and is used by the EU for vaccine licensure purposes
	ELISA	Measures influenza-specific IgM, IgG and IgA subclasses; measures both neutralizing and non-neutralizing antibody
Anti-neuraminidase serum antibody	Neuraminidase inhibition	Enzyme-linked lectin assay is gaining popularity and could be standardized among laboratories to promote studies to identify anti-neuraminidase antibody as an immune correlate for reduced disease
Anti-M2 antibody	Peptide ELISA; flow cytometry or cell-based ELISA	Indirect ELISA using synthetic peptide may not detect potentially protective antibodies recognizing conformational M2 epitopes. Assays that detect antibodies to expressed M2 proteins offer a promising alternative
IgA at mucosal surface	ELISA	Sensitive, standardized, qualified assays and standardized sample collection methods are needed to improve detection and evaluation of this response as an immune correlate of protection
Serum cytokine	ELISA	Quantifies specific cytokines in serum but does not measure the type or number of cytokine-producing cells
CD4 T cell	Cellular proliferation	May be useful in combination with other measurements in flow cytometry-based techniques
	ELISpot	Measures both function and number of responding T cells. Efforts have been made to standardize granzyme B and IFN-Y ELISpot
	Flow cytometry	One of the most common methods used to quantitate cellular function. Ability to measure a range of T-cell phenotypes and functions and quantify the number of responding cells in multiplex format
	Tetramer staining	Limited use due to technical difficulties in developing HLA class II tetramers; complicated by genetic polymorphism
CD8 T cell	Cellular proliferation	May be useful in combination with other measurements in flow cytometry-based techniques
	Cytotoxicity assays	Measure functions important to control of influenza, but quantification of the number of cytotoxic effector cells is difficult
	ELISpot	Measures both function and number of responding T cells. Efforts have been made to standardize granzyme B and IFN-Y ELISpot
	Flow cytometry	One of the most common methods used to quantitate cellular function. Ability to measure a range of T-cell phenotypes and functions and quantify the number of responding cells in multiplex format
	Tetramer staining	Complicated by genetic polymorphism
B cell	ELISpot	Quantifies the number of B cells secreting antigen-specific antibody; may detect responses to LAIV

ELISpot: Enzyme-linked immunosorbent spot; LAIV: Live-attenuated influenza vaccine.