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Human Vaccines & Immunotherapeutics

ISSN: 2164-5515 (Print) 2164-554X (Online) Journal homepage: https://www.tandfonline.com/loi/khvi20

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To cite this article: Cécile van Els, Siri Mjaaland, Lisbeth Næss, Julia Sarkadi, Eva Gonczol, Karen Smith Korsholm, Jon Hansen, Jørgen de Jonge, Gideon Kersten, Jennifer Warner, Amanda Semper, Corine Kruiswijk & Fredrik Oftung (2014) Fast vaccine design and development based on correlates of protection (COPs): Influenza as a trendsetter, Human Vaccines & Immunotherapeutics, 10:7, 1935-1948, DOI: 10.4161/hv.28639

To link to this article: https://doi.org/10.4161/hv.28639

Human Vaccines & Immunotherapeutics 10:7, 1935–1948; July 2014; © 2014 Landes Bioscience

Fast vaccine design and development based on correlates of protection (COPs)

Influenza as a trendsetter

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Keywords: correlates of protection, influenza, COP based vaccine design, vaccine development

Abbreviations: ADCC, antibody-dependent cell-mediated cytotoxicity; COP, correlate of protection; HA, hemagglutinin; IIV, inactivated influenza vaccine; LAIV, live attenuated influenza vaccine; M1, matrix protein; M2, ion channel protein; M2e, extracellular domain of M2; NA, neuraminidase; NP, nucleoprotein; PA, polymerase A; PB, polymerase B; WIV, whole inactivated virion

New and reemerging infectious diseases call for innovative and efficient control strategies of which fast vaccine design and development represent an important element. In emergency situations, when time is limited, identification and use of correlates of protection (COPs) may play a key role as a strategic tool for accelerated vaccine design, testing, and licensure. We propose that general rules for COP-based vaccine design can be extracted from the existing knowledge of protective immune responses against a large spectrum of relevant viral and bacterial pathogens. Herein, we focus on the applicability of this approach by reviewing the established and up-coming COPs for influenza in the context of traditional and a wide array of new vaccine concepts. The lessons learnt from this field may be applied more generally to COP-based accelerated vaccine design for emerging infections.

Introduction

At the global level several factors contribute to a significantly increased risk of appearance and spreading of new and re-emerging infectious diseases. Global warming influences the geographical distribution of important vector organisms for several dangerous pathogens[1,2] and the increase in worldwide travel and mobility is an effective driving force for the spread of infectious diseases. The unexpected appearance of Severe Acute Respiratory Syndrome (SARS) in 2002[3] and the related Middle East Respiratory Syndrome (MERS) in 2013,[4] the pandemic H1N1 influenza virus in 2009[5] and the continued threat of H5N1 and H7N9 bird flu,[6,7] serve as examples of emerging and re-emerging diseases, that require swift control measures.

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Submitted: 12/05/2013; Revised: 03/14/2014; Accepted: 03/24/2014; Published Online: 04/24/2014; http://dx.doi.org/10.4161/hv.28639

The potential threat from emerging and dangerous infections calls for innovative and efficient control strategies in order to increase the general preparedness level. When facing an emergency situation with a new pathogen, time is limited and efforts to accelerate the otherwise time-consuming vaccine development process at all stages are critical. The use of correlates of protection (COPs) may play a key role in speeding up vaccine development from design to licensure in various ways. In this context we define COPs as immune responses that directly can be linked to protection in a causal way, whereas surrogates or co-correlates are understood as responses co-occurring with protection but not necessarily representing a causal mechanism.

In the design phase, vaccine composition and delivery systems may be selected to elicit known or presumed COPs. In the clinical phase, vaccine efficacy measurements performed by monitoring early biomarkers in validated assays instead of measuring protection against clinical disease may provide a faster, but still reliable, outcome. COPs may also be used to bridge preclinical protection studies performed in animal models with clinical studies, or connect early with late phase trials, supporting a faster and more evidence-based progress toward licensure. If it is not feasible to assess clinical efficacy, the size and duration of phase 3 trials can be reduced by using COPs to predict vaccine efficacy.

To be able to apply COP-based vaccine design to new and re-emerging infections for which COPs have not yet been discovered, the establishment of general predictive rules based on the most important classes of pathogens is needed. This can be achieved by systematic extraction of the existing knowledge of protective immune responses against a large spectrum of relevant bacteria and viruses combined with knowledge of the immune mechanisms induced by different vaccine concepts. To demonstrate the applicability of this principle, we have reviewed the current literature on COPs for an infectious disease for which traditional vaccine platforms are being constantly improved and multiple novel vaccine concepts have reached clinical trials: influenza. The 2009 influenza pandemic reminded us that

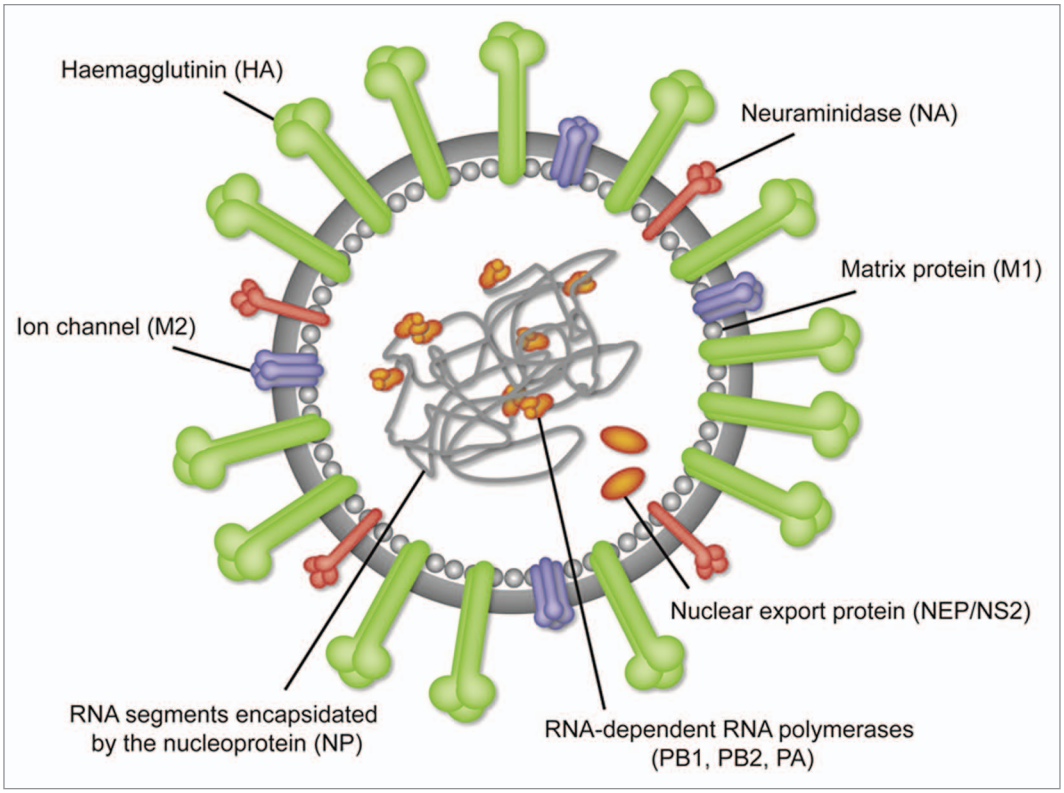

Figure 1. Nomenclature and localization of the major external and internal protein antigens of the influenza virus.

several general aspects of vaccine development are important to improve pandemic preparedness. Among the most important are the needs for universal vaccines comprising conserved antibody and T-cell targets to induce cross-reactive immunity in all age groups, improved adjuvants, and vaccine production platforms which allow fast manufacture. Recently proposed novel COPs for influenza are mostly based on clinical data with sufficient power to support prediction of protection. Importantly, the development of these COPs which goes far beyond the traditional hemagglutination inhibition (HI) assay paves the way for the rational design and faster evaluation of new and promising vaccine concepts. We suggest that the recently obtained knowledge in this field, as summarized herein, is not only important for improved vaccination against influenza, but due to its unique size and scope can also be generalized and applied to accelerate vaccine design and development for a wider range of new and reemerging infections.

Classical Influenza Vaccines and Traditional COPs

Influenza viruses are enveloped viruses that contain a segmented genome of 8 different negative sense single-stranded RNA molecules. The envelope and its 3 integral membrane proteins hemagglutinin (HA), neuraminidase (NA) and the ion-channel M2 overlay a matrix protein (M1), which encloses the virion core. In this core the ribonucleoprotein (RNP) complex, which consists of the viral RNA segments coated with nucleoprotein (NP) and the RNA-dependent RNA polymerases (PB1, PB2, and PA) are found (**Fig. 1**).

Seasonal influenza is dominated by A and B viruses. Influenza A viruses are divided into subtypes according to the combination of different HA and NA antigens occurring at the surface. Among many possible subtypes of influenza A viruses (17 HA and 9 NA subtypes), H1N1 and H3N2 are the most predominant subtypes currently circulating among humans. Seasonal influenza is associated with the annually occurring sequence variation in HA and NA (antigenic drift), whereas influenza pandemics usually occur as a result of new combinations of HA and NA subtypes (antigenic shift). Due to the continuous antigenic drift of HA (and NA), the composition of virus-derived vaccines against seasonal influenza must be updated on an annual basis to achieve the necessary matching between HA antibody specificity and the prevalent circulating influenza strains.

Two main types of vaccines for seasonal influenza are currently licensed: inactivated influenza vaccines (IIV) mostly for intramuscular administration, and live attenuated influenza vaccines (LAIV) for intranasal delivery. Both vaccine types are usually trivalent comprising 2 A subtypes (currently H1N1 and H3N2) and a single B component, yet quadrivalent vaccines containing an additional B component now also emerge on the market (**Table 1**). Inactivated vaccines may contain whole inactivated virus particles (WIV vaccines), virus disrupted by detergents or solvents (split vaccines) or purified HA and NA (subunit vaccines).

Split and subunit vaccines are today the most frequently used products and represent the conventional vaccine concepts for

Table 1. Approved seasonal and (pre)pandemic influenza vaccines

Vaccine purpose strain	Vaccine type	Adjuvant	Route	Trade names[1] (manufacturer)
Seasonal annual strains[2]	IIV whole virus	AlPO4	im	Fluval AB (Omninvest)
	IIV split	none	im	FluLaval[3] (ID Biomedical)
		none	im	Fluarix, Fluviral (GlaxoSmithKline)
		none	im	Enzira[4] (Pfizer)
		none	im	Vaxigrip, Fluzone[3] (Sanofi Pasteur)
		none	im	Afluria[4] (Merck)
		none	im	GC FLU (Green Cross Corporation)
		none	id	Intanza, IdFlu, Fluzone Intradermal (Sanofi Pasteur)
		none	im	Fluvax[4] (bioCSL)
	IIV subunit	none	im	Influvac, Imuvac, Fluvaccinol (Abbott)
		none	im	Agriflu, Agrippal, Fluvirin,[4] Optaflu[5] (Novartis)
		none	im	Mutagrip (Sanofi Pasteur)
		MF59C.I	im	Fluad (Novartis)
		none	im	FluCelvax[4,5] (Novartis)
		virosomal lipids	im	Inflexal® V[4] (Berna Biotech, Crucell)
	LAIV	none	in	Fluenz, Flumist seasonal[3] (MedImmune)
	Recombinant protein	none	im	Flublok®[6] (Protein Sciences)
Pandemic 2009 H1N1 A/Cal7/2009	IIV whole virus	none	im	Celvapan H1N1[5] (Baxter)
		AlPO4	im	Fluval P (Omninvest)
	IIV split	none	im	Panvax[4] (Merck)
		none	im	Panenza (Sanofi Pasteur)
		none	im	Green Flu-S (Green Cross Corporation)
		AS03	im	Pandemrix (GlaxoSmithKline)
	IIV subunit	none	im	Fluvirin-H1N1[4] (Novartis)
		MF59C.1	im	Celtura,[5] Focetria (Novartis)
	LAIV	none	in	Fluenz, Flumist pandemic (Medimmune)
		none	in	Nasovac (Serum Institute of India)
Pre-pandemic various avian strains	IIV whole virus	AlPO4	im	Fluval H5N1 (Omninvest)
		AlOH3/PO4	im	Daronrix (GlaxoSmithKline)
		none	im	Celvapan H5N1,[5] Vepace[5] (Baxter)
	IIV split	AS03	im	Prepandrix, Adjupanrix (GlaxoSmithKline)
	IIV subunit	MF59C.1	im	Foclivia, Aflunov (Novartis)

[1]Vaccines are derived from processed influenza virions propagated on embryonated chicken eggs and inactivated using formaldehyde unless otherwise indicated [2]; seasonal vaccines are mostly trivalent (2 A strains and 1 B strain) unless otherwise indicated [3]; available as quadrivalant vaccine (2 A strains and 2 B strains); [4]β propiolactone virus inactivation; [5]cell based virus propagation; [6] high dose trivalent uncleaved HA (rHA0) based on baculovirus expression in insect cells.

seasonal vaccination. LAIV most often consist of cold adapted and temperature sensitive viral strains, and have been licensed both in US (2003) and Europe (2011). There are indications that LAIV may be more efficacious in young individuals (6 mo to 18 y), whereas no apparent difference in efficacy of LAIV and IIV has been demonstrated in adults (reviewed in[8]).

While most IIV are non-adjuvanted, a few have been optimized by improved formulations to generate better protection both among the elderly[9] and young children,[10] who typically respond poorly to vaccination. Manufacturing of classical influenza vaccines may further vary with respect to virus propagation procedures and inactivation methods. For (pre)pandemic purposes, several IIV and LAIV vaccines have been licensed, some of which are cell based and/or adjuvanted, and widely used in various age groups during the 2009 influenza pandemic. Recently, as a first non-classical influenza vaccine, a recombinant baculovirus expressed trivalent HA0 based vaccine (FluBlok®) was introduced on the market, a technological advance which may speed up mass production of vaccines for novel viral strains[11] (**Table 1**).

Minimal requirements for traditional seasonal influenza vaccines

While annual updating of an already licensed vaccine in many cases does not include immunogenicity testing, introduction of a new seasonal influenza vaccine requires a licensing application which should include satisfactory evidence of immunogenicity and safety as laid down by the regulatory authorities (EMA in Europe and FDA in USA). The humoral responses induced in the required trials are currently evaluated with the traditional "gold standard" COP for influenza: titer of antibodies inhibitory for the major surface antigen hemagglutinin (HA).[12] HA is the viral receptor-binding protein, and antibodies that are directed to epitopes located within or in close proximity to the receptor-binding site, can prevent binding of the virus to its receptor on the host

Table 2. Novel influenza vaccine approaches in clinical development categorized according to the primary mode of protection intended to be induced, vaccine type, and antigen targets

Novel concepts: major mode of protection[1]	Vaccine type (Ag)	Examples of concepts in clinical development	Ref.[2]
More broadly reactive antibodies	Recombinant protein or synthetic polypeptide-based vaccines (HA, NP, M1)	• Multimeric-001, purified recombinant protein (*E. coli*) containing 9 conserved epitopes from HA, NP, and M1, non-adjuvanted or ISA-51, i.m. (BiondVax)	140
	DNA vaccines (HA, NP, M2e, NA)	• Monovalent (H5) or trivalent (H5-NP-M2) plasmid DNA, formulated with Vaxfectin® adjuvant, i.m. (Vical Inc.) • VGX-3400,[3] NA, M2e, NP, and subtype specific HA regions, i.d. and i.m. electroporation (Inovio) • HA expressing plasmids adjuvanted with DEI-LT (DNA encoded Immunostimulator-lanile Toxin) on gold particles, epidermal (PowderMed)	141 118, 30 142
	Particulate vaccines: virosomes, VLP,[4] BLP[5] (HA, NA, M1)	• HA, NA, M1 VLP produced in insect cells by baculovirus infection, i.n. (NovaVax) • Alhydrogel® formulated H5-VLP produced via the bacterial vector *A. tumefaciens* in tobacco leaves, i.m. (Medicago) • Matrix MTM adjuvanted H5N1 virosomes (Berna, Crucell) • FluGEM®, IIV antigens mixed with self-adjuvanting BLP from inactivated *L. lactis* bacteria, i.n. (Mucosis)	30, 60 143 144 112 (6)
	Alpha virus-based vaccines (HA)	• H3 expressed in propagation-defective, single-cycle RNA replicon α virus vector system producing virus-like replicon particles (VRP), i.m. (AlphaVax)	145
	Heterologous fusion constructs (HA cleavage site, NP, M2e)	• Qbeta VLP, M2e fused to HB core particle, spontaneously packaging bacterial RNA carrying ligands for TLR7 and TLR3, i.n. (CYTOS Biotechnology) • M2e and HAcleavage peptide-OMPC construct with alum or proprietary adjuvant, i.m. (Merck) • M2e-Flagellin[7] fusion construct (VAX102) i.m. (VaxInnate) • ACAM-FLU, recombinant M2e-HB core Ag fusion construct, non-adjuvanted, alum or QS21, i.m. (Acambis) • N8295: M2e-NP fusion protein covalently linked to CpG, i.m. (Dynavax)	110 71,30 146-148 147, 30 72
Cross-reactive T cell immunity	Vector delivery of Ag MVA, adenovirus (HA, NP, M1)	• MVA-NP-M1, replication defective MVA virus, i.m. or i.d. (Impfstoffwerk, Jenner Institute) • Ad4-H5-Vtn, oral, also induce antibody responses (PaxVax) • ChAdOx1 NP+M1, i.m, (Clinical Biomanufacturing Facility, University of Oxford)	91 95 96
	Peptide based: conserved epitopes (NP, M1, M2)	• Flu-v, ISA-51 adjuvanted synthetic vaccine containing 6 conserved CTL epitopes (NP, M1, M2), s.c. (SEEK) • Flunisyn™ (FP01), depot forming nanoparticles with 6 conserved T cell peptide epitopes fused to a fluorocarbon tail, i.m. (Immune Targeting Systems, ITS)	93 ITS website
Mucosal sIgA immunity	LAIV	• LAIV NS1-deletion, HA altered cleavage, i.n., also induce T cell immunity (Green Hills)	30
	Mucosal recombinant viral vector delivery (HA)	• Recombinant (replication defective) Ad5 delivery of HA, i.n. (Vaxin) • Recombinant (replication defective) Ad5-TLR3 ds RNA adjuvanted delivery of H5, oral (Vaxart)	113 114
	Mucosal non-replicating vaccines (HA)	• HA split antigens mixed with neisserial OMP (FluINsureTM), i.n. (GSK-ID Biomedical)	111
	Mucosal delivery systems	• Nasal, oral, sublingual, needle-free dermal administration, mucosal adjuvants	31

[1]In addition to the major mode of protection, several of the vaccine concepts also induce other types of immunity: all DNA vaccines, particulate vaccines, α virus vaccines, Multimeric-001, LAIV and viral vector delivery vaccines also induce T cell immunity; [2]In addition to references, information is also taken from the websites of companies; [3]preclinical data for VGX-3400; [4]VLP, viral like particle; [5]BLP, bacterial like particle; [6]preclinical data for FLU-GEM®; [7]Flagellin-platform is also used for potentiating the induction of subtype specific HI titers to H1 (VAX125, VAX128), H5, and H7.

cells. It is well documented that the induction of HA specific antibodies is an important COP against infection, provided that these antibodies have the correct strain-specificity.[13] Therefore, the induction of HA-antibodies that block receptor-binding is used as a correlate of vaccine efficacy and a current criterion for regulatory approval. Pre- and post-vaccination sera obtained in the registration trials from subjects of different age groups are tested, mostly in the hemagglutination inhibition (HI) assay. In this assay, inhibition of virus-induced agglutination of red blood cells by HA antibodies in serum samples can be measured as the HI titer. Based on studies with seasonal influenza A strains in adults, an HI titer ≥ 40 has been defined as an immunologic correlate corresponding to a 50% reduction of risk of contracting influenza.[12,13] For a seasonal vaccine to be registered it must meet, for each tested age group and all viral strains, at least one of 3 defined criteria for immunogenicity as evaluated by the HI assay: the sero-protection rate, the sero-conversion rate, and the mean HI titer increase. Notably, for registration of pandemic vaccines all 3 criteria must be fulfilled.

Limitations of using HI as COP for evaluation of influenza vaccines

Although an HI titer ≥ 40 to a specific influenza strain remains indicative of protection, evidence is accumulating that the relevance of the HI assay may differ substantially between different vaccines and populations tested. First, in the human challenge model it has proved difficult to infect individuals with HI titers as low as ≥8,[14] and LAIV show protective efficacy independently of HI titers. Being administered intranasally, LAIV will prime local mucosal immune responses and secretory IgA, not measured in the HI assay, but most likely contributing to protection.[15] Second, poor sensitivity of the HI assay has been found for neutralizing antibodies that recognize the conserved HA stalk region, as well as for antibodies specific for avian A virus subtypes H5 and H7, and for B strains, warranting technical modifications.[16,17] Third, the use of the adult correlate of protection (HI titer ≥ 40) may not be appropriate when evaluating vaccination of children. As reported by Black et al. a cutoff of 110 was required to predict the conventional 50% clinical protection rate, and to predict an 80% protective level, which would seem more desirable from a public health perspective, a titer of 330 was required.[18] This discrepancy may in part relate to the ability of the HI assay to measure both IgM and IgG antibodies, which may lead to an 'overrating' of the immune response in naïve children,[19] and measuring IgG only may therefore correlate better with protection in pediatric trials. On the other hand, alternative immunological endpoints, such as antibody responses to other viral proteins or cell mediated immune parameters, might better reflect vaccine efficacy in children.[19-21] In fact, vaccine-induced T-cell responses have been suggested as a better COP than antibody responses not only in young children,[22] but also in adults over 65 y of age.[23] The primary goal of vaccination, specifically in the elderly, is to provide protection against clinical disease, rather than sterilizing immunity. Thus, enhanced vaccine-mediated protection against influenza illness in older individuals should probably be assessed by changes in cellular immunity, in addition to standard HI measurements.[24]

These and other studies have increased the awareness among scientists, vaccine manufacturers and regulatory authorities that new COPs are needed, not only for improved evaluation of traditional influenza vaccines in different target groups, but also for the development of new vaccine concepts.[19-21,25,26] Various new seasonal and (pre)pandemic vaccine approaches use novel production- or formulation platforms that do focus on inducing strong(er) HI titers to the immuno-dominant globular head(s) of HA subtypes,[27-29] such as FluBlok®.[11] For other new vaccine concepts in clinical development, the traditional HI titer may not serve as a relevant COP at all. Dependent on the antigens targeted, such as conserved domains of HA, NA, M2e, or universal T-cell antigens (like NP, PA, PB, or M1), and dependent on their formulation or expression platform,[27-39] other COPs should be addressed. These include broadly virus neutralizing activity, antibody dependent cellular cytotoxicity (ADCC), cross-reactive T cell responses, or mucosal secretory IgA (sIgA). As is the case for serological assays,[40,41] the application of any cellular biomarkers of protection will require standardized and harmonized assays with inactivated or low pathogenicity viral strains and criteria to compare results between laboratories. Although a wide range of potential COP assays covering both antibody- and cell-mediated immune responses are in the pipeline, they still need further evaluation for clinical performance and methodological validation.

Concomitantly, a diverse landscape of upcoming influenza vaccine approaches has boosted the exploration of new immunological end-points to be used for assessing vaccine immunogenicity and effectiveness of immune responses. In a broader context, this development is not only important for influenza, but the lessons learnt from this field may also pave the way for rational development of vaccines for other diseases based on the same principles. Thus, the putative trendsetting role of influenza can in this regard be attributed to both development of principally new vaccine concepts and a substantial broadening of the repertoire of COPs necessary for efficient evaluation of novel vaccines in general.

Influenza Vaccine Concepts Requiring New COPs

A variety of innovative influenza vaccine approaches in clinical development, that are not solely based on HI assays, broadly target one or more of the following principles of protection: (1) induction of more broadly cross-reactive serum antibodies, (2) cross-reactive cellular immunity, and/or (3) mucosal immune responses (**Table 2**). Potential new influenza COPs relevant for these different vaccine platforms have been identified from preclinical and clinical studies, as reviewed from various perspectives.[20,21,25,42,43] Although some immunological biomarkers only can be considered as co-correlates or surrogates, and not true COPs, they may still play a role in evaluating vaccine candidates.[44] In children, serum HI titer and nasal IgA[45] have been described as co-correlates. In addition, non-neutralizing antibody- and T-cell responses have also been reported as co-correlates of protection.[46] Nevertheless, despite the complexity of

Table 3. Established and emerging COPs for influenza vaccine development

COP assay immune parameter Ag specificity	Mode of protection for immune response	Examples of relevant vaccine concepts	Status of COP assay and comments
HI assay **Serum IgG** **HA globular head**	Preventing intracellular infection to be established: Inhibition of binding to cell surface receptor	WIV, split, subunit Virosomes Recombinant Ag DNA vaccines	Well established as COP (EMA, FDA)[12,13] Limit for protection defined (HI titer > 40) Variation with virus subtype and target age group Standardized but still large variability between labs.
HA ELISA **Serum IgG** **HA stem region**	Preventing intracellular infection to be established: Virus neutralization Inhibition of fusion with the endosomal membrane	Recombinant protein or polypeptide based Ag with relevant HA sequences	Experimentally demonstrated[47-49] More data needed Conserved among subtypes Limit for protection not defined Relevant for universal vaccines
NA ELISA and NAI assay **Serum IgG** **NA**	Preventing mature virus to be released after intracellular infection: Inhibition of NA enzyme activity	LAIV WIV, split, subunit, Virosomes	Experimentally established[25,42,43,56,57] Broadly cross-reactive non-neutralizing antibodies Limit for protection not defined
VN assay **Serum IgG** **HA, NA**	Neutralizing virus: Preventing intracellular infection to be established	LAIV WIV, split, subunit, Virosomes	Experimentally demonstrated[25,42,43] More data needed Limit for protection not defined
M2e ELISA **Serum IgG** **M2e**	Not virus neutralizing but reducing virus spread after intracellular infection (ADDC)	Recombinant fusion proteins: M2e sequence coupled to TLR ligands or in immunogenic particles	Demonstrated in animal models[73] More data needed Conserved among subtypes Limit for protection not defined Relevant for universal vaccines
IgA ELISA **Mucosal sIgA** **HA, NA**	Preventing virus to enter the body by neutralization at mucosal surfaces	Mucosally delivered vaccines: LAIV, WIV, and recombinant viral vector based delivery	Experimentally demonstrated[45,99] More data needed Limit for protection not defined
Cytotoxicity assays[1] **CD8+ and CD4+** **T cell cytotoxicity** **M1, M2, NP, PA, PB**	Preventing severe clinical disease and death when infection is established by lysing infected cells	LAIV, WIV Recombinant fusion protein, synthetic (poly)peptide or vector-based vaccines covering T cell epitopes	Experimentally established[45,99] Granzyme B assay: standardized, validated[79,97] Flow based CD107a assay and Cr51 release assay used in a human challenge model[83] Lower limit for protection not defined Relevant for universal vaccines
INFγ ELISPOT[1] **INFγ pos. cells** **M1, M2, NP, PA, PB**	Preventing severe clinical disease and death when infection is established	LAIV, WIV Recombinant fusion protein, synthetic (poly)peptide or vector-based vaccines covering T cell epitopes	Experimentally demonstrated in humans[22] Frequency of INFγ producing cells IFNγ+IL2- CD8+ phenotype[77] Standardization possible by commercial kits Proposed limit for protection: 100 SFC/mill. PBMC Relevant for universal vaccines
Cytokine assays1 **Cytokine profiles** **M1, M2, NP, PA, PB**	Preventing severe clinical disease and death when infection is established by helping (other) T- and B cell functions	LAIV, WIV Recombinant fusion protein, synthetic (poly)peptide or vector-based vaccines covering T cell epitopes	Experimentally established in humans[23,97] IFNγ/IL10 ratio: standardized, validated assay[23,97] IL-21 production[81] Lower limit for protection not defined Relevant for universal vaccines

[1]Require cell isolation and (depending on study design) cryopreservation.

the protective immune response, a good understanding of the underlying mechanisms is fundamental to accelerate the development of any novel influenza vaccine concepts. Importantly, when applied in a broader context of vaccinology, this knowledge may also play an important role in rational development of vaccines against other diseases.

Broadly cross-reactive antibody responses to envelope structures as COPs

Antibody responses elicited toward HA primarily bind to the hyper-variable globular head region and only recognize homologous strains within a given virus subtype. This is why the efficacy of conventional seasonal influenza vaccines is mostly limited to

circulating strains with a close antigenic match. More broadly reactive antibody responses seem to be directed at less accessible epitopes spanning the interface of properly folded HA trimers, or at the more membrane proximal stalk region of the HA molecule. This stalk region of HA is relatively conserved and is involved in fusion with the endosomal membrane of the host cell. Broadly neutralizing antibodies that bind to this region have been shown to inhibit infection with influenza A[47-49] and B strains.[50] To target these less variable HA regions more efficiently than conventional virus derived vaccines, several expression platforms delivering full-length HA have entered clinical development. These include recombinant and fusion-proteins, particles (inactivated virions, virosomes, VLPs, BLPs), replicon systems, and DNA vaccines (**Table 2**). Recently, a phase I trial of an intramuscularly given H5 DNA prime, split vaccine boost regimen provided evidence for the induction of anti-stalk antibodies in humans.[51] New approaches only targeting the stalk region showed feasibility in preclinical studies, but these concepts have not yet entered clinical development.[52-54] Serological assays, such as ELISA and a pseudotype neutralization assay[49,55] may be used to assess the induction of HA stalk specific antibodies (**Table 3**).

The other major viral envelope protein and component in various novel influenza vaccine approaches is NA. Its function as a sialic acid cleaving enzyme is crucial for efficient release of virions from infected cells, acting in the late stage of the virus replication cycle. Antibodies directed against the enzymatic site can block its function and serum anti-NA antibodies have been associated with resistance to clinical disease.[56,57] Hence, unlike antibodies against HA which can prevent infection, NA-specific antibodies limit the release and further spread of viral particles, and thereby mainly prevent serious disease and fatal outcome. Although NA antibodies are considered mostly as subtype specific, they also have cross-reactive potential.[58,59] Clinically tested vaccine approaches including NA involve DNA based vaccines[30] and particulate forms, such as virus like particles (VLP)[30,60] (**Table 2**). Notably, these platforms can also induce various forms of cellular immunity. The induction of serum NA inhibiting (NI) antibodies is suggested to be an independent predictor of immunity to naturally occurring influenza in the presence of HI antibodies.[61] Various non-standardized methods are available to assess anti-NA antibody responses,[62-65] including assays that minimize steric hindrance by anti-HA antibodies.[57,66,67]

The M2 protein is a tetrameric membrane protein that forms an ion channel and plays an important role in the un-coating of the viral genome during the early steps of the virus replication cycle. M2e (the extra cellular domain of M2) is highly conserved in all influenza A subtypes. M2-specific antibodies tend to recognize virus particles poorly, do not neutralize the virus, and antisera against virus contain few M2-specific antibodies. However, M2e is highly expressed in infected cells, and anti-M2e antibodies binding to these cells can induce antibody dependent NK cell mediated killing (ADCC), complement-mediated lysis, or phagocytosis.[68,69] As the M2-protein is conserved across influenza A strains, it has been regarded as a basis for the development of universal vaccines.[34,70] Despite the fact that neither M2e seroprevalence after recurrent infections with circulating influenza strains nor vaccination with LAIV or WIV vaccines indicate a strong immunogenicity profile, this vaccine concept is still pushed in the clinical pipeline[38,48,71,72] (**Table 2**). Assays to evaluate M2e antibody responsiveness may be based on linear peptides or conformational tetrameric forms of the M2e antigen.[73]

Besides the traditional HI assay, several other antibody-based assays, as mentioned here, have been demonstrated to play an important role in evaluating new vaccine concepts in the development process (**Table 3**). However, any general assessment of their relative strength and value as potential COPs in clinical trials is complicated, due to the fact that their relevance will be dependent of several factors: vaccine concept to be tested, immune responses to be targeted, and even the population to be vaccinated. Thus, the broad repertoire of COP related assays now available in the influenza field, including both humoral and cellular immune responses, may serve as a necessary menu from which the relevant immune assays should be chosen.

Cross-reactive T cell responses as COPs

In contrast to neutralizing antibodies directed mainly against the highly variable surface antigens (HA and NA) of the influenza virus, cellular immune responses mediated by T cells are mainly directed against epitopes of internal and highly conserved antigens (M, NP, PA, PB). Antibody-mediated immunity mainly acts to prevent viral entry into and release from cells, while T-cell mediated immunity plays an important role in clearance of established infection and reduction of disease severity and mortality. Although the complementary protective role of T-cells has been known for a long time,[74] it has recently been highlighted by the demonstration of pre-existing T-cell immunity against the 2009 H1N1 pandemic strain, leading to protection against severe disease in the absence of specific antibody responses.[75]

Cellular effector functions related to protection can be mediated by 2 conventional T cell subsets. Cytotoxic CD8+ T cells recognize peptide epitopes generated from cytosolic viral proteins by proteasomal degradation and presented by MHC class I molecules on the surface of infected cells. Upon activation, these T cells secrete antiviral cytokines and release perforin and granzymes, of which the latter initiate apoptosis of the infected target cell. In a prospective study low granzyme B levels correlated with the risk of developing influenza disease in older adults.[76] More recently levels of pre-existing IFNγ+IL2- CD8+ T cells were shown to correlate with low total symptom score during the 2009 pH1N1 pandemic.[77] Importantly, granzyme B levels were also shown to correlate with protection and enhanced cytotoxic activity after influenza vaccination.[23,78-80] CD4+ T cells, on the other hand, recognize viral epitopes that have been processed in the endo-lysosomal pathway of antigen presenting cells and presented by MHC class II molecules. These cells act not only by orchestrating the responsiveness of CD8+ T cells and B cells by secreting Th1 (IFNγ, TNFα), Th2 (IL-4, IL5, IL13), Th17 (IL-17), Tfh (IL21), or regulatory (IL-10, TGF-β) cytokines,[81] but also by directly killing infected cells.[82] Pre-existing influenza-specific CD4+ T cells were recently found to correlate with decreased viral shedding and reduced severity of illness following experimental influenza infection of humans.[83] In addition, a decline in

the INF-γ/IL-10 ratio produced by PBMC stimulated in vitro with influenza virus was shown to be associated with increased risk for influenza disease in the elderly.[23] Induction of CD4+ T cell immunity may correlate with the serological response to influenza vaccination: the CD4+ Th1 response after primary H5N1 vaccination predicted protective antibody levels after booster doses,[84,85] and frequencies of IFN-γ and IL-17 producing CD4+ T cells correlate with HI responses in younger adults after seasonal vaccination.[86] While influenza specificT cell responses seem particularly relevant as COPs[22,23,78] and indeed can contribute to cross-protective immunity,[87] tight regulation is needed to avoid immuno-pathological effects after vaccination as described for natural infection.[88-90]

Of the most widely used seasonal influenza vaccines, split and subunit vaccines elicit CD4+ T cells specific for epitopes from HA and NA subtypes, while LAIV can also induce CD4+ and CD8+ T cell responses to epitopes from internal proteins. Multiple vaccine candidates using liver vectors or particulate formulations are in clinical development specifically designed to induce cross-reactive T cell immunity against internal antigens or their epitopes[91-96] (**Table 2**). For these and several other novel platforms that deliver viral antigens into the MHC class I or II processing pathway, either as recombinant live attenuated virus, recombinant synthetic polypeptide, DNA, α-virus, or as formulated particles (**Table 2**), standardized CD8+ and CD4+ T cell assays are needed to assess vaccine effectiveness and to compare results between different laboratories (**Table 3**).

Granzyme B is a key mediator of cytolytic activity of CD8+ T cells and an assay based on its enzymatic activity (serine protease) has been developed[79] and recently standardized and validated by several laboratories.[97] This assay warrants application to evaluating protective T-cell immunity in clinical trials. To probe the different functional cytokines released by CD4+ T-helper cells that support the induction of cellular immune responses (INF-γ, TNF-α, and IL2), promote antibody responses (IL4, IL5, IL13), or play a regulatory role (TGF-β, IL10), various assays are available. Multicolor flow cytometry has the advantage of combining the characterization of cell surface markers and intracellular production of multiple cytokines at the single cell level. Based on this approach various subsets of multifunctional T cells have recently been proposed as predictors of protection.[77,81,83] Besides standardized ELISA assays, it is also possible to measure a wide range of secreted Th1 and Th2 cytokines in multiplexed assays following standardized and validated formats.[97] Based on the established correlation between the INF-γ /IL10 balance and protection against disease,[23] such assays should represent efficient tools for evaluating vaccines in combination with assays for cytotoxic effector functions (granzyme B assay). The relevance of INF-γ has been further supported by the finding that the frequency of virus specific INF-γ secreting cells could be correlated with protection against disease in a vaccination study of children by using an ELISPOT assay.[22]

The relative strength of the T cell based COPs discussed here is difficult to evaluate, since they are proposed on the basis of clinical studies with different design performed in various age groups, and only some of them are supported with data from animal models. Nevertheless, the evidence for CD8+ T cell mediated cytotoxicity as a valid COP is strong, due to a large number of human and animal studies employing different experimental settings and technical assays to demonstrate the relevance of this effector function.[23,43,74,76,79,98] Among the cytokine based COPs suggested, there are reasons to consider INF-γ positive T cells as a relevant correlate based on preclinical and clinical studies involving both controlled vaccine efficacy trials and population based observational studies.[22,23,77] Although the recently reported protective role of T cell subsets expressing defined combinations of cytokines and surface markers seems promising,[77,81,83] the experimental evidence and relative strength for such assays as COPs, so far may be assessed as somewhat weaker.

Mucosal immune responses as COPs

The principal advantage of mucosal immunization is the induction of local immune responses at the site of infection, in addition to systemic immunity. As recently reviewed,[99] various immune mechanisms at the mucosal surface of the human respiratory tract may act to prevent or clear influenza infection. It is well established that secretory IgA (sIgA) released at mucosal surfaces, as well as plasma derived IgG can control viral infection in the respiratory tract, but sIgA has a greater potential to cross-protect against heterologous influenza strains.[100,101] Mucosal IgA is capable of neutralizing virus once inside mucosal epithelial cells.[102] Local intracellular virus may also be cleared by cytolysis of infected cells by intra-epithelial cytotoxic CD8+ T cells, and vaccine induced NK cell mediated mechanisms have also been implicated in viral clearance. The superiority of LAIV over IIV noted in various meta-analyses of vaccine efficacy in different study populations (not only limited to children), is partly attributed to their differential capacity to induce mucosal antibodies in the respiratory tract.[103-105] IIV are injected intramuscularly and induce good serum antibody responses but no mucosal antibodies, whereas intranasally administered LAIV efficiently prime both virus-specific mucosal IgA and serum IgG responses.[106] Mucosal immune mechanisms are mediated by the mucosa-associated-lymphoid tissue (MALT), a system of anatomically separate but functionally connected mucosal lymphoid sites, such as the nasopharynx-associated lymphoid tissue (NALT) and the gut-associated lymphoid tissue (GALT).[107,108] Successful priming at mucosal surfaces requires an appropriate type of vaccine antigen (pathogen derived complex particles), and in case of subunit antigens a mucosal adjuvant system is usually also necessary.[109] Vaccine platforms most successful in activating mucosal immunity are either attenuated pathogens (like LAIV), replicating vectors administered to mucosal tissues, or vaccines that mimick mucosal pathogens in particle size, innate properties and the ability to target mucosal dendritic cells (DC).[108,110] Delivery routes that are effective at inducing mucosal immune responses in combination with novel influenza vaccine platforms include intranasal,[30,60,110-113] oral,[95,114] sublingual,[115-117] and intradermal[118] administration. Some of these concepts are already in clinical development (**Table 2**).

An increasing body of evidence suggests that IgA responses contribute to protection against influenza, and this parameter

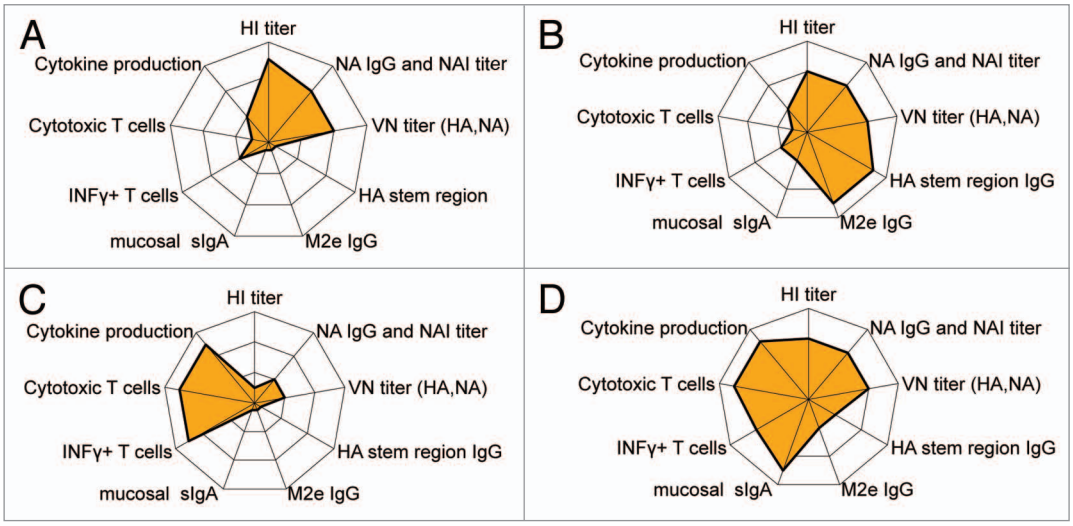

Figure 2. Connectograms showing both type and strength of a spectrum of immune responses induced by principally different influenza vaccine concepts: (**A**) Classical split or subunit vaccines mostly inducing vaccine specific HA and NA antibodies, (**B**) Vaccines specifically targeting a more broad antibody response with potential for cross-protection, (**C**) Vaccines specifically targeting conserved antigens for cross-reactive T cell responses, and (**D**) LAIV covering a broad range of both humoral and cellular immune responses.

should be further evaluated as a valid COP, at least for mucosal vaccines. At present measurement of sIgA in mucosal secretions such as nasal lavages is not standardized, neither with regard to clinical sampling nor assay methodology.[42] Current methods are mostly based on IgA ELISA,[107,119] and the results were found to correlate to HI and VN titers in nasal lavage[120] but not in serum. In **Figure 2** we have illustrated the broadening of protective immune responses to influenza by highlighting some vaccine concepts in development compared with traditional split or subunit vaccination.

Systems Vaccinology: A New Approach for Discovery and Application of COPs in Vaccine Development

Systems vaccinology represents an entirely different approach which is already showing promise for establishing as yet unknown COPs for several diseases.[121-124] Developments in transcriptomics, proteomics, and bioinformatics have made it possible to perform genome-wide and global analysis of host responses after infection and vaccination, allowing for discovery of new and relevant correlates or surrogates of protection. Systems vaccinology aims to identify both innate and adaptive immune signatures able to predict the immunogenicity and protective capacity of vaccines, as well as identification of potentially novel mechanisms of immune regulation. For instance, the outcome of LAIV or IIV vaccination in man with respect to the humoral response could be predicted based on the expression of a defined set of genes only 3 d after vaccination.[121] Such systems biology approaches could be useful, not only to shorten clinical testing but also to assess the quality of animal models and identify animal vs. human correlates. A substantial portion of the recently obtained knowledge from systems vaccinology is based on influenza work. We therefore claim that the rapidly moving influenza field strongly contributes to improved understanding of novel biomarkers for protection that may be generally applied to accelerate vaccine development processes.

Animal Models and Bridging to Human COPs

The successful use of animal models for human vaccine development largely lies in the ability to extrapolate virus and vaccine induced immune response, virus behavior and virus induced clinical disease and pathology from animals to humans. A number of small animal models exist for preclinical evaluation of influenza vaccines of which the 2 mostly used model systems are mice and ferrets.[125]

To be able to measure COPs, the availability of a relevant challenge model remains crucial. To this end, observations and analyses made in infected humans and clinical challenge trials[83] should guide the choice of animal model and outline the parameters to be monitored and measured in a preclinical study. For influenza infection, these parameters include influenza like illness, pathology, mortality, virus susceptibility, replication kinetics and organ distribution, as well as virus transmission. Despite the widespread use of mice, the clinical disease and pathology seen in the mouse influenza model do not correlate well with observations in humans.[125-127] Moreover, mice are not susceptible to most human seasonal strains, which require adaptation, and the outcome of challenge studies performed in mice should therefore be treated with caution. On the other hand, ferrets develop fever, nasal discharge, suffer from weight loss and develop lung pathology and thus more closely resemble infection in humans. Ferrets also exhibit the same subtype-dependent severity of disease which can vary from a common cold to a deadly disease.[125-128] Moreover, ferrets are susceptible to human and avian influenza

strains, largely show the same tissue distribution of infection, and are able to spread the virus through aerosol transmission. This means the ferret is a suitable model to study the effect of vaccines on influenza virus transmission which is particularly important for universal vaccines that most likely will not provide neutralizing protection. Additional factors that should be considered when setting up a challenge model are the route of infection[129] and the infectious dose.[130] Intranasal administration of H5N1 influenza results in a severe CNS disease and intratracheal administration in severe pneumonia[129] of which only the latter has been described in humans.[131,132]

The immune system that most closely resembles humans is that of non-human primates. Although this model is used for some influenza studies it is not the model of widespread choice, due to ethical and economic reasons.[125,128] Preclinical research is therefore based on models that to a lesser extent represent the human immune system, but still may be good predictors. Antibody responses can be measured equally well in mice and ferrets by means of ELISA (IgG and IgA), HI, NI, and virus neutralization. The mouse model, however, offers greater opportunities for measuring cellular immune responses. The availability of inbred, knockout and transgenic mouse strains and the feasibility of adoptive transfer and T-cell depletion experiments greatly enhance the investigation of COPs. Such experiments have demonstrated a role for CD8+ T cells in viral clearance and contributed to establishment of immune correlates in mice. Furthermore, single epitope vaccination and flow cytometric analysis of epitope specific INF-γ producing CD8+ T cells has even revealed the contribution of a single T-cell clone to virus control.[98] In addition, the availability of HLA-transgenic mice increases the relevance of this research for humans.[133,134] These types of experiments are not feasible in ferrets since they are outbred animals, the nature of their MHC is unknown, and cellular reagents are scarce. Nevertheless, some cellular reagents, developed for other species, do show cross-reactivity with some of the CD markers and cytokines in ferrets, which allows the detection of CD3, CD8, and INF-γ.[135,136] In addition, INF-γ ELISPOT assays have been developed but are not yet commercially available.[137] Moreover, innate cytokine responses have been measured at the mRNA level by RT-PCR.[138]

Owing to their wide availability, the range of immunological reagents available, easy handling and cost benefits, inbred mice models are typically used as the first model of choice for proof of concept, dose ranging studies, and understanding of COPs. Subsequently, the ferret model is exploited to further investigate the protective effectiveness of a vaccine. Recent developments allowing basic analysis of cellular immune responses in ferrets should ultimately reveal whether these responses correlate with those in humans. If so, this could also shed light on the extent to which murine COPs can be translated to clinical correlates. Although care should be taken,[99] utilization of biomarkers for vaccine effectiveness in ferrets for development of human COP assays is regarded as an obligatory and strategic step in influenza vaccine development.

Concluding Remarks

For a range of diseases, markers of an appropriate humoral immune response have so far been the most frequently used and best validated correlates of protective immunity after vaccination. However, technological and conceptual advancements within the field of cell-mediated immunity have led to a number of new immunological read-outs with the potential to emerge as valuable correlates of vaccine-induced protection. Whereas antigen-specific production of the Th1 cytokine IFN-γ has been considered as a quantitative marker of protective immunity, recent evidence from several infectious disease models has pointed to multifunctional CD4+ T cells as an even better correlate of sustained protective immunity.[139] In addition, new technological possibilities to measure antigen-specific cytotoxic CD8+ T cell responses and cytokine patterns after infection or vaccination, has opened a new vista to include defined T-cell mediated effector functions in the evaluation of protective immune responses. In this context, the influenza field plays an important role as a trendsetter for a range of other viral and even bacterial diseases by guiding the development of similar COPs needed for fast design and development of new vaccines against emerging infections.

While HI assays still represent the most important COPs for evaluation and licensing of traditional influenza vaccines, new vaccine concepts targeting other antigens, delivery systems, and protective mechanisms have paved the way for several new and emerging COPs with relevance for both antibody and T-cell mediated immunity (**Fig. 2**). Specifically, the possibility to utilize conserved T-cell antigens (NP, M2, PA, and PB) and conserved HA and NA domains in universal vaccination against seasonal and pandemic influenza, highlights the need for introducing COPs that are relevant to these immune responses. Although the relative importance of the novel COPs reviewed here is difficult to rank, we now have a spectrum of relevant assays available with probably different strength for being used as a predictor of protection in different settings. Hence, the application and practical value of each COP assay may depend on the type of vaccine to be used as well as the immune responses and age groups targeted. Although several new and potential important COPs for influenza have been introduced and successfully applied in vaccine development, there is still a need for validation and standardization of such assays, including definition of protective levels, to secure their optimal use and interpretations in comparative analysis. This will also be a prerequisite before these assays can be adopted by regulatory authorities for approval purposes.

Importantly, research on influenza vaccines and COPs has not only paved the way for improved understanding of protective immune responses against influenza infection and disease, but also of which upcoming and principally different vaccine platforms that can induce the appropriate immune responses. Finally, results from the influenza field have taught us how to measure the corresponding and relevant laboratory-based COPs at the methodological level. All these aspects represent important contributions to the knowledge-based platform we need for rapidly developing efficient vaccines against emerging infections. By

systematically extracting and processing the same knowledge elements from a spectrum of viral and bacterial infections, it should be possible to establish general predictive rules for optimal and fast vaccine design based on COPs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was conducted as part of "FASTVAC: A generic framework for FAST production and evaluation of emergency VACcines," a project funded by the Health Program of the European Union. Drs Elly van Riet, Willem Luytjes, and Nynke Rots are kindly acknowledged for advice and Mrs Anke Smits for helping with the manuscript.

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