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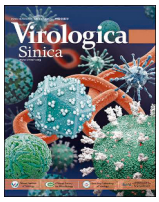

Review

Structural vaccinology expedites rational design of next-generation vaccines for influenza and respiratory syncytial virus

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ABSTRACT

Vaccination stands as the single most effective and cost-efficient public health intervention in human history, serving as a cornerstone of modern medicine that profoundly transforms global health outcomes. Beyond preventing disease, it acts as a catalyst for equitable socioeconomic development. In recent decades, recurrent seasonal viral outbreaks and sporadic yet catastrophic pandemics have continued to pose challenges to global public health systems. Traditional vaccine technologies, however, not only often fall short in protection efficacy, but also fail to keep pace with the evolving demands of next-generation vaccine development. These scientific gaps have directed cutting-edge research to prioritize critical objectives in terms of enhancing antigen effectiveness, achieving stable pan-protection against diverse variant strains, and strengthening production robustness. The advent of genomics spurred the emergence of reverse vaccinology 1.0, leading to breakthroughs like the MenB vaccine. Today, the advanced reverse vaccinology 2.0 paradigm thoroughly redefines vaccine design process by organically integrating human immunology with state-of-the-art computational protein structure analysis tools. This review explores the transformative shifts in influenza and respiratory syncytial virus vaccine development, along with specific case studies, to deepen understanding of the evolving principles and methodologies in novel vaccine designs and offer strategic insights for addressing emerging infectious pathogens.

INTRODUCTION

The past 50 years have witnessed the Expanded Programme on Immunization delivering historic achievements, the eradication of smallpox, the near-elimination of polio, and curbing measles spread and so on, collectively saving at least 154 million lives and embodying a holistic approach to health (Samarasekera, 2024; Wahl and Pitzer, 2024; Mirza et al., 2025). The history of vaccinology is a narrative of relentless innovation, fueled by defining critical scientific questions of each era. From the empirically rooted inoculation to the revolutionary advances in reverse vaccinology fueled by genomics, the evolutionary course of vaccinology consistently harbors scientific lessons that signal limitations in vaccine research. The recurring influenza virus evasion and the vaccine-enhanced respiratory disease (ERD) that haunted early respiratory syncytial virus (RSV) vaccination efforts have profoundly reflected the inherent incapacity of traditional vaccine strategies to tackle such challenges (Kapikian et al., 1969; Petrova and Russell, 2018; Polack et al., 2021; Han et al., 2023; Azbazdar et al., 2025). In such contexts, cross-disciplinary structure-based rational antigen engineering has already emerged as an inevitable solution to address these long-standing challenges in vaccine development. By mapping neutralization epitopes and clarifying immunodominance hierarchies, adaptive immunological investigations lay the groundwork for identifying novel targets to devise effective immunization strategies (Mclellan, 2015; Kwong and Mascola, 2018; Lim et al., 2022; Wang, A. et al., 2022; Fan et al., 2024; Sun et al., 2024; Song et al., 2025). With the target defined, protein engineering technology precisely modifies antigens at an atomic level, allowing them to selectively display stable neutralizing epitopes and induce desired host immune responses (Dormitzer et al., 2008; Patel et al., 2023).

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As a subfield of vaccinology, structural vaccinology has established rational, systematic methodologies and frameworks for novel vaccine design (Dormitzer et al., 2008). The research process encompasses solving high-resolution structures, identifying protective human B-cell epitopes, and engineering target epitopes, all of which aim at refinement of vaccine antigen stability, immunogenicity, protection efficacy as well as safety (Nguyen et al., 2025). Isolation and characterization of potent human broadly neutralizing antibodies (bnAbs) not only contribute to defining critical epitopes but also aid in stabilizing metastable target antigens for X-ray crystallography (Mclellan et al., 2013a). Structural vaccinology-enabled precise optimization has led to breakthrough vaccines against RSV, influenza, human immunodeficiency virus (HIV), and other pathogens, providing a powerful strategy to tackle viral infections that are refractory to traditional vaccination or with no currently available vaccines. In influenza vaccine development, structure-based rational vaccine design surmounts limitations of traditional strain prediction by targeting conserved conformational epitopes amenable to neutralization, paving the way for universal protection (Group et al., 2013; Deng et al., 2015; Morris et al., 2018; Wei et al., 2020). By defining and stabilizing key antigenic regions, researchers engineered diverse novel immunogens with enhanced breadth and potency of immune responses, moving the field beyond the need for annual updates toward durable universal influenza vaccines. The application of structural vaccinology in the RSV vaccine field successfully addressed a major historical setback of the ERD caused by earlier formalin-inactivated RSV (FI-RSV) vaccines. By mapping critical neutralization-sensitive epitopes and introducing stabilizing amino acid substitutions, researchers created stabilized pre-fusion (pre-F) protein antigens that elicit potent and safe immune responses (Swanson et al., 2020; Chang et al., 2022; Che et al., 2023; Lee et al., 2024; Liang et al., 2024), demonstrating how structural insights can turn past failures into clinical successes. This review elaborates particularly on the rational design processes of novel antigens as a core stage in vaccine development, so as to help subsequent designers quickly formulate design concepts and engineering strategies.

A BRIEF HISTORY OF VACCINOLOGY DEVELOPMENT

As early as China's Western Han Dynasty, the philosophical reflection of preventive medicine in *Huangdi Neijing* puts forward “Superior physicians prevent disease rather than cure it”. This was translated into empirical prophylactic practice via China's sophisticated variolation, which was documented no later than the 16th century (Jiang, 2008), standardized as “ripe pox” variolation and formally codified in 1742 as part of the medical work *Yizong Jinjian*. This empirical inoculation, which involved nasal exposure to smallpox scab or pustule, effectively reduced smallpox mortality in recipients.

Modern vaccination originated in 1796, when Edward Jenner validated vaccinia inoculation for smallpox protection, building on observations of smallpox resistance in dairy maids exposed to cowpox. Though not the first vaccinia attempt, Jenner's investigations on cowpox vaccines as a pioneering example of evidence-based medicine revolutionized scientific thought and discovery approach by demonstrating the value of empirical observation combined with clinical validation, laying the groundwork for immunology and translational medicine. Known as Jennerian vaccination, this technique henceforth gradually replaced risky human variolation worldwide, shifting the smallpox control and driving its 1977 eradication (Riedel, 2005; Jenson et al., 2016). However, early rudimentary arm-to-arm inoculations led to the inadvertent transmission of blood-borne diseases including syphilis and tuberculosis, which brought unforeseen public health burdens (Imperato and Henderson, 2010). This was mitigated, particularly by the retro-vaccination, which yielded relatively safer inocula by passaging human-infectious vaccinia strains through susceptible animals (Copeman, 1898).

Microbiology advances in the late 19th century, notably the confirmation of the germ theory via *Bacillus anthracis* by Robert Koch in 1876, in turn ushered in Louis Pasteur's vaccine era (Manchester, 1995; Rekvig, 2024). Pasteur's seminal work in the 1880s demonstrated that immunization with weakened or killed pathogens including chicken cholera, anthrax and rabies can effectively ward off pathogen-associated diseases, which scientifically validated acquired immunological memory as the basis for vaccination (Mostowy, 2022). These scientific contributions cemented the germ theory of disease and founded modern vaccinology in the 20th century. Among landmark vaccines from the legacy of these foundational insights are the Salk inactivated polio vaccine licensed in 1955, and the Sabin oral polio vaccine granted licensure in 1961, with the latter oral delivery further streamlining population immunity establishment (Salk, J. and Salk, D., 1977; Almond et al., 1987; Smith and Leggat, 2005; Bakker et al., 2011; Li and Wang, 2025), and the Bacille Calmette-Guérin (BCG) vaccine developed at the Pasteur Institute, which represents yet another critical validation of Pasteur's theoretical discoveries (Cho et al., 2021; Starshinova et al., 2025). Advances in immune biology, pathogen cultivation, purification techniques, and later genetic engineering, collectively spurred a rapid surge of novel vaccine platforms (Kaufmann, 2019), including the attenuated, inactivated, toxoid-based vaccines, polysaccharide conjugate, subunit protein, and protein nanoparticle vaccines. The World Health Organization's Expanded Program on Immunization, launched in 1974, has further boosted health outcomes and resource utilization cost-effectively (Mirza et al., 2025). As vaccine-related theory grows increasingly sophisticated and the range of diseases it addresses expands, it has developed into the interdisciplinary and standalone vaccinology. Overall, effective vaccines developed during this stage were primarily composed of whole pathogens and natural molecules, identified through empirical screening rather than rational design, which generally hindered the mechanistic elucidation and resolution of specific vaccine defects.

In the late 1990s, Rino Rappuoli introduced the reverse vaccinology paradigm that integrates bacterial whole-genome sequencing and computational biology (Rappuoli, 2000; Sette and Rappuoli, 2010; Caugant and Brynildsrud, 2020), most notably solving the unmet vaccine need for *Neisseria meningitidis* serogroup B (MenB), with the landmark achievement of Bexsero, a recombinant multicomponent protein-based vaccine. MenB's capsular polysaccharide shares structural homology with human antigens, making it a historically unsuitable target for polysaccharide-based approaches applied to meningococcal serogroups ACWY and *Streptococcus pneumoniae* (Nedelec et al., 1990; Coquillat et al., 2001; Masignani et al., 2019). This portable novel approach was later applied to bacteria, fungi, and parasites research (Rappuoli, 2000; Weichhart et al., 2003; Serruto et al., 2009; Sette and Rappuoli, 2010; Schubert-Unkmeir and Christodoulides, 2013), informing investigational pneumococcal vaccines and global tuberculosis vaccine concepts (Knaust and Frosch, 2004; Masomian et al., 2020; Watt and Liu, 2020). Subsequently, stringent precision epitope design has been regarded as essential for the development and optimization of vaccines for intractable RNA viruses, such as HIV, influenza, dengue, SARS-CoV-2, and hepatitis C virus (HCV). For example, dengue vaccines can trigger antibody dependent enhancement, similar to early FI-RSV vaccines yet driven by distinct immunopathological mechanisms. The reverse vaccinology 2.0 technological framework has been increasingly recognized as vital for this purpose, integrating human immunology-guided antigen design, computational protein analysis, and artificial intelligence assistance to generate vaccine antigens tailored to pre-specified protective immune profiles (Burton et al., 2012; Rappuoli et al., 2016; Wang, J. et al., 2022; Bakkers et al., 2024b; Koh et al., 2025; Villanueva-Flores et al., 2025). RSV pre-F and COVID-19 spike vaccines showcase the immense potential of structure-based rational design to control protein folding and antigenicity at the atomic level. Yet designing vaccines to prevent immune escape in frequently mutated viruses with limited conserved epitopes remains a daunting task. This article primarily focuses on the guidelines for target selection and methods of structural stabilization in novel antigen design.

BREAKING THE STRAIN-SPECIFICITY BARRIER: DEVELOPING NEXT-GEN BROADLY NEUTRALIZING INFLUENZA VACCINES

Influenza A and B viruses within the *Orthomyxoviridae* family represent a persistent global public health threat. Their eight-segment negative-sense RNA genomes encode at least 17 and 11 viral proteins, respectively (Vasin et al., 2014; Chauhan and Gordon, 2022). Influenza A viruses pose the highest risk due to zoonotic transmission, emergence of highly pathogenic variants, and pandemic potential (Mallapaty, 2024). Major pandemics including 1918H1N1, 1957H2N2, 1968H3N2, and 2009H1N1 caused severe global disease and death (Monto and Fukuda, 2020). Antigenic shifts drive pandemic emergence, whereas antigenic drift sustains seasonal epidemics, creating a long-term challenge for prevention. Developing a universal influenza vaccine is therefore a high priority, given its ability to provide broad, durable protection and diminish the need for annual immunization.

Elucidating structural basis of hemagglutinin (HA) allostery during infection is important for understanding immuno-focusing strategies and rational antigen engineering. Influenza viruses initiate infection primarily through the binding of the major envelope glycoprotein HA to sialic acid receptors on respiratory epithelial cells, with seasonal strains preferentially recognizing α-2,6-linked Neu5Ac (Liu et al., 2022; Chien et al., 2023). HA is synthesized as the precursor HA0, which requires cleavage into HA1 and HA2 subunits to enable conformational rearrangements essential for viral infectivity (Fig. 1A) (Skehel and Wiley, 2000; Das et al., 2018). As a class I fusion protein, HA trimers undergo precisely regulated structural transition cascade upon endosomal acidification, allowing the HA2 fusion peptide to mediate viral-host membrane fusion (Das et al., 2018). A pH of typically 4.8–5.6 triggers early HA refolding by altering protonation of some conserved histidine residues that can act as acidity sensors and disrupting associated salt bridges (Milder et al., 2022; Badiee et al., 2024). Recent study reveals that HA2-mediated fusion is more of a dynamic multi-step process rather than a one-step "spring-loaded" event, with long-lived dynamic intermediates observed on virions (Benhaim et al., 2020). These intermediate ensembles are thought to persist until sufficient fusion machinery accumulates forming a matrix and driving efficient membrane fusion. During dramatic structural rearrangement, the heptad repeat A-helix and the interhelical B-loop extend into an elongated coiled-coil, while part of the membrane-proximal β-sheet (HA2 residues 23–36) unfolds to form a flexible linker that connects the extended coiled-coil to the fusion peptide, directing the fusion peptide toward the target cellular membrane (Benton et al., 2020; Garcia-Moro et al., 2022). This low pH-induced transition largely dismantles broadly neutralizing epitopes in the HA stem, leaving merely a small fraction of protective epitopes intact in the postfusion conformation (Finney et al., 2024).

Inducing strong and long-lasting broadly sterilizing immunity is regarded a critical attribute of a universal influenza vaccination strategy (Krammer et al., 2018; Nachbagauer and Palese, 2020). Previous studies have demonstrated that preexisting anti-HA stem antibody responses are associated with cross-protective immunity against influenza in the general population (Park et al., 2018). Their neutralizing protection mechanism no longer relies on serum hemagglutination inhibition that is widely considered an accepted immune correlate of protection of seasonal influenza vaccines, but rather on the suppression of viral membrane fusion (Corti et al., 2017; Liu, X. et al., 2025). Anti-HA stem antibodies may also facilitate the elimination of virus-infected cells that display viral HA on their surface through Fcγ receptor-mediated effector functions, such as antibody-dependent cellular cytotoxicity and antibody-dependent cellular phagocytosis (Dilillo et al., 2014, 2016; Deng et al., 2018). A wealth of scientific evidence supports the notion that the broadly neutralizing efficacy largely depends on the preservation of epitopes within the HA stem in prefusion state (Sautto et al., 2018; Nachbagauer and Palese, 2020; Wei et al., 2020). This holds true

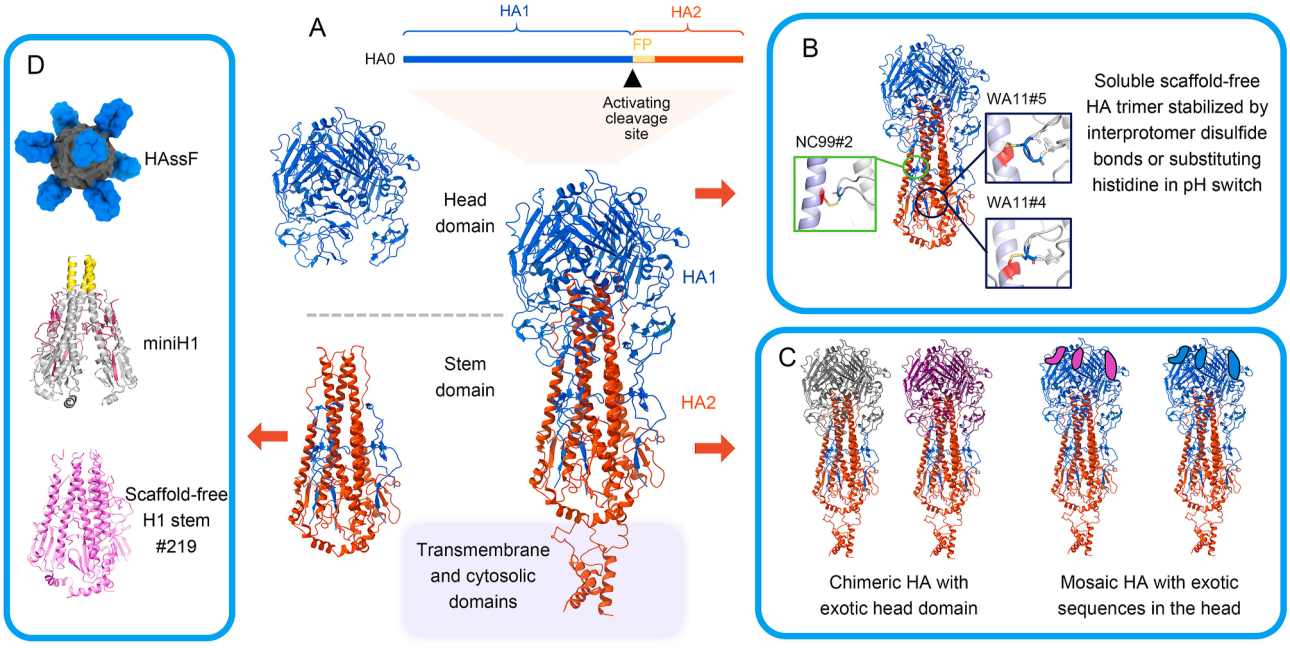

Fig. 1. Design strategies for universal influenza vaccines. **A** Schematic diagram of influenza virus HA polypeptide and prefusion HA structure. The folded HA1 polypeptide mostly forms the head domain; a minor part forms the stalk domain with the HA2 polypeptide. FP denotes the fusion peptide. This full-length HA structure model is derived from the solved structure model with PDB ID 6HJR. **B** Design strategy for soluble HA trimeric protein with transmembrane and cytoplasmic domains removed (Liu et al., 2024b). The interprotomer disulfide bonds are formed by cysteine residues on two separate protomers, which are shown in red and blue, respectively, in close-up stick model views. **C** Construction of head-swapped chimeric HA antigen and mosaic HA antigen designs. Head domains and spots therein in either navy blue or red colors denote peptide fragments derived from exotic avian influenza viruses. **D** Universal influenza vaccine antigens designed based on the HA stem domain. The structural design of HAssF features eight HA stem trimer antigens (blue) displayed on the self-assembling ferritin nanoparticle core (grey) (Yassine et al., 2015). In the structural diagram of the miniH1 stem trimer (Impagliazzo et al., 2015), the yellow-labeled α-helix at the apex corresponds to the exogenous GCN4 motif fragment.

to all design innovations as described below. It is well established that the immune system preferentially targets the hypervariable head domain of HA, facilitating viral immune evasion via antigenic drift in head domain. In contrast, the stem domain, despite its remarkable pan-group conservation, generally remains immuno-subdominant due to steric occlusion imposed by the head domain (Wu and Wilson, 2017; Liu, C.C. et al., 2025). Redirecting or subverting the immunodominance hierarchy by maximizing the engagement of cognate B-cell receptors (BCRs) with conserved epitopes from the HA stem has long remained a central goal in vaccine design.

Compared to stem-only approaches, full-length (FL) HA-based manipulation offers a viable path with greater assurance of structural stability. The computationally optimized broadly reactive antigens (COBRAs) approach employs genetic algorithms and layered consensus-building steps to generate composite HA sequences. It consolidates multiple HA sequence clusters from extensive and diverse intra-subtype strain data into a single broadly cross-reactive antigen (Allen and Ross, 2021; Nunez et al., 2021). This approach uniquely preserves HA epitope characteristics across historical and modern viral strains, eliciting head-specific neutralizing immunity that confers broad intra-subtype cross-protection, and overcomes HA drift-mediated antigenic variation. For instance, the H1 subtype COBRA rHA X6 exemplifies this strategy by retaining intact head and stem epitopes representative of both historic and recent H1 isolates and demonstrates broad intra-subtype cross-protection (Nagashima et al., 2024). Yet, the marked subtype specificity of the antigenicity of the immunodominant HA head and limited stem-directed immune responses confine COBRA to intra-subtype protection. Another strategy indicated that structural repositioning of the stem overcame steric hindrance. Immunizations with nanoparticles displaying inverted HA trimers generated a broadly protective antibody response that was preferentially directed toward epitopes within the stem (Frey et al., 2023; Xu et al., 2024). Ferritin is a stably self-assembling protein nanoparticle exhibiting octahedral symmetry, featured by a tractable structure where the N-termini of three subunits located along its triple axis of symmetry are externally exposed and relatively spatially proximate. By genetic fusion with HA at the N-termini of each subunit, eight HA trimers are presented on the surface of the ferritin nanoparticle (Kanekiyo et al., 2013). The fixed 70° angle between the three-fold axes of symmetry in the regular octahedron, along with the fixed distance of over 10 nm between the HA molecules, provides sufficient room for cognate BCRs to access stem epitopes. Experimental results demonstrated that vaccination with ferritin-HA nanoparticles considerably enhanced cross-neutralizing protective efficacy.

Building on these insights, we hypothesized that soluble FL HA antigens, which conceivably greatly improve the stem exposure, could substantially enhance the induction efficiency of stem-specific antibodies. To engineer scaffold-free soluble HA proteins, we strategically introduced cysteines for forming interprotomer disulfide bonds via iterative approaches (Fig. 1B) (Liu et al., 2024b). It was during the research we subsequently discovered that negative-stain electron microscopic observations revealed the stem structure of the HA generated by this approach is more compact compared with HA trimers dependent on exogenous trimerization motifs, and antigenicity assessment assays also indicated that its stem possesses comprehensive native antigenicity. In the course of engineering, high-resolution HA structures informed the selection of pairs of interfacial residues meeting stringent geometric criteria, including opposing α carbon (Cα)-Cβ vectors with optimal Cα-Cα distances not more than an empiric value of 7 Å to accommodate disulfide bond formation. Each candidate was subjected to iterative *in silico* validation as follows. Ramachandran plots confirmed permissible backbone dihedral angles for cysteine substitutions, followed by rotamer ensemble sampling to identify side-chain configurations that meet ideal disulfide bond parameters, such as a distance of around 2 Å for two putative sulfur atoms and proper dihedral angle $\chi 3$ within $90 \pm 30°$ (Haworth et al., 2007; Ramanujam et al., 2019), and conformation dynamics simulation to exclude visible steric clashes, a tripartite filtering process ensuring native-like trimer integrity. While disulfide-stabilizing effects were observed in optimal HA trimer constructs, it is also noted that the efficiency of artificial disulfide bond formation might be affected by drifted HA sequence variants in a somewhat inexplicable manner. This scientific phenomenon has also been revealed by structural comparison of wildtype and mutant proteins determined by X-ray crystallography, even a few point mutations at discrete loci can trigger long-range structural perturbations that alter folding dynamics (Mclellan et al., 2013a).

This design process shows general portability across influenza A (H1, H3), and B HA, yielding two to three stabilized variants per subtype with preserved both inter- and intra-protomer antigenicity from initial libraries containing dozens of variant candidates. Mouse immunization studies revealed appreciable redirection of antibody responses toward the stem. For instance, vaccination with the soluble disulfide-stapled H3 trimer antigen elicited a serum IgG response with 13% being stem-specific, a proportion significantly higher than that induced by the seasonal influenza vaccine (Liu, C.C. et al., 2025). We established a robust and generalizable workflow integrating computational design with empirical validation, which provides an optional approach for engineering stable soluble homo-oligomeric antigens. Consistent success across phylogenetically distinct influenza subtypes, along with methodological rigor as evidenced by an acceptable 11% optimal candidate ratio for wet lab validation, supports broad applicability for vaccine design. Additionally, the developed scaffold-free HA backbone may be further rationally optimized in conjunction with the COBRA methodology to tackle influenza viruses exhibiting HA drift.

Vaccines based on FL HA, including quadrivalent influenza vaccines (QIV), are capable of inducing stem-targeted memory B cell responses, but there is a general trend toward a reduced relative proportion of memory B cells with stem specificity overall after administration with the QIV booster, such weak specific immunity is hard to function as independent correlate of cross-protection (Abreu et al., 2020). Sequential immunizations with chimeric HA (cHA) antigens represent an effective strategy for the induction of broad influenza protection (Fig. 1C) (Bernstein et al., 2020; Nachbagauer et al., 2021). The conception of this technical solution is rooted in two immunological phenomena, original antigenic imprinting and immuno-focusing on conserved epitopes. These engineered cHAs are constructed by combining head domains derived from antigenically distinct zoonotic subtypes with a conserved stem backbone (Hai et al., 2012; Liu et al., 2019; Puente-Massaguer et al., 2023; Yi et al., 2025). By leveraging human immunological naivety to exotic head epitopes, this approach boosts immune responses directed at the stem region. Structural studies by Rong Hai et al. identified a conserved intra-protomer cysteine bridge (Cys52–Cys277) across subtypes, hinting a structural boundary in between two domains, nearby which the head replacement can be executed properly (Hai et al., 2012). Interestingly, these cHAs retain fusogenic activity, with slightly lower replication efficiency versus wild-type viruses, supporting the viable production of cHA-displaying live-attenuated, inactivated, and virus-like particle vaccines. A phase I clinical trial showed that the vaccination regimen with the cH8/1 live-attenuated vaccine followed by either the non-adjuvanted cH5/1 inactivated vaccine or AS03-adjuvanted cH8/1 and cH5/1 inactivated vaccines effectively elicited cross-protective anti-stem IgG responses (Bernstein et al., 2020; Nachbagauer et al., 2021). Animal immunization studies demonstrated that sequential immunizations with cH9/1, cH8/1, and cH11/1, all of which integrate HA heads derived from diverse avian influenza strains, robustly reactivated H1 stem-specific memory B-cell responses and conferred cross-neutralizing immunity against heterosubtypic H5N1 (Rathnasinghe et al., 2024). Heterologous HA imprints among populations across different ages may influence the cross-protective efficacy of the sequential immunization regimens. Overall, these findings pave a way for developing more intricate and effective strategies.

A sequential-based alternative for broad influenza protection is the mosaic HA (Fig. 1C), which is a more advanced and sophisticated iteration of the cHA vaccine. Computationally designed based on sequence data, mosaic HA replaces and silences the major HA inhibition-reactive antigenic sites in the head instead of the entire domain using site-corresponding sequences from exotic avian HA strains that are rarely isolated in human populations (Sun et al., 2019). During the design process, genetic algorithms are utilized to optimize, screen, and perform "mosaic" splicing to generate several optimized sequences that are non-existent in nature (Kamlangdee et al., 2014). Experimentally selected HAs then incorporate refined T-cell epitopes, achieve higher expression, and form stable trimers that retain native structure (Broecker et al., 2019; Liu et al., 2023). This strategy redirects immune responses from the variable immunodominant epitopes on the HA head to the stem region as well as other conserved regions including subdominant head epitopes, thereby promoting broadly reactive antibody response by utilization of more diverse germline gene origins (Broecker et al., 2019; Sun et al., 2019; Liu et al., 2023; Liu, X. et al., 2024; Zhao et al., 2025). This mosaic approach is especially valuable for the influenza B HA, aside from its successful application in influenza A HAs. The B cHA head domain is typically also derived from exotic avian influenza A HAs and fused with the B virus stem, but viable viruses expressing B cHA cannot be rescued, potentially due to functional incompatibility between the A virus head domain and the B virus stem (Sun et al., 2019). In contrast, the mosaic approach successfully rescues viable viruses with B/mHAs, in which the HA head immunodominance is ablated (Sun et al., 2019; Liu et al., 2021). Additionally, no significant differences are expected when using different B HAs as backbones due to the lack of subtypes and high sequence similarity beyond replaced variable major antigenic sites. Sequential immunization experiments in mouse models showed that B/mHA vaccines conferred cross-protection against both homologous and heterologous influenza B virus strains. Both the host restriction that humans are their sole natural reservoir and the low evolutionary rate of B/HA allow for the possibility of eradicating influenza B viruses. An increasing number of studies have discovered broadly neutralizing antibodies against conserved head regions across diverse subtypes (Jiao et al., 2023). Cognate conserved epitopes within those sequentially used cHAs presumably contribute to elevation of cross-reactive antibody levels, with the receptor-binding site pocket typically being the most conserved (Yoshida et al., 2009; Ohshima et al., 2011; Ekiert et al., 2012; Krause et al., 2012; Mccarthy et al., 2018; Li, T. et al., 2022), while occluded epitopes hidden on the HA head interface elicit antibodies largely with cross-group breadth (Bajic et al., 2019; Bangaru et al., 2019; Watanabe et al., 2019; Dong et al., 2020), and broadly neutralizing antibodies that bind the vestigial esterase subdomain merely possess subtype-specific neutralizing activity (Li et al., 2009; Tan et al., 2016; Smith, G. et al., 2017; Bangaru et al., 2018; Yan et al., 2021).

Featuring conserved, pan-protective epitopes across nearly its entire structure, the HA stem-only antigen is widely regarded as an ideal candidate for efficiently developing broadly protective immunity via immuno-focusing. HA stem-only antigen design grapples with hurdles involving structural refactoring to retain the essence of supersite and immune refocusing on them. Removing transmembrane domain (TM) disrupts trimerization of HAs from many strains, what's worse, head deletion unsettles native interfacial interactions and exacerbates stem misfolding. The stability of prefusion HA stem homotrimer hinges primarily on adequate interprotomer interaction energy. Structural investigations have flagged stability-critical areas and residues, uncovering hotspots for stabilizing substitutions (Cotter et al., 2014; Byrd-Leotis et al., 2015; Hanson et al., 2015). Seminal studies reported in 2015 achieved groundbreaking stabilization of native-like H1 stem antigens through iterative designs, followed by elucidating their trimeric architecture (Fig. 1D) (Impagliazzo et al., 2015; Yassine et al., 2015). In the process of engineering miniH1, initially, an appropriately sized linker replacing the head domain allows the remaining peptides of HA1 at both sides have room to maintain original spatial positions and then a disulfide bond established between this flexible linker and the adjacent long helix compensates for the conformational entropy increase of the remaining fragment caused by head removal. Elimination of the cleavage site in fusion peptide via substituting the arginine residue robustly restricts the extent of low pH-triggered coiled coil extension (Chen et al., 1998; Garcia-Moro et al., 2022). The construction process reveals that extensive optimization of newly exposed B-loop residues that interact with the head in FL HA contributes to reduced aggregation and increased solubility. A standout finding is replacing the membrane-distal C-helix portion with a truncated GCN4 motif, which structurally docks within a heptad repeat; a further disulfide bond establishment between this motif and B-loop repairs the defective three-stranded coiled coil structure, enabling trimer formation of miniH1. Yet such forced trimerization typically observes quaternary conformational torsion to a slight degree at the stem domain, which might not be perfect for the induction of protective antibodies (Milder et al., 2022; Liu et al., 2024b). Immunization experiments with animal models demonstrated that the best-performing construct miniH1 conferred effective cross-protection against heterosubtypic H5N1 infection. Of greater note, recombinant *Helicobacter pylori* ferritin self-assembling nanoparticles presenting HA stem antigens derived from both group 1 and group 2 displayed more prominent immunogenicity (Corbett et al., 2019; Moin et al., 2022). Clinical trials involving H1ssF and H10ssF have demonstrated immuno-focusing efficacy targeting the stem and cross-neutralizing protection in humans (Widge et al., 2023; Casazza et al., 2024). Meticulously modified ferritin scaffolds in HAssF antigens induced strong specific immune responses, yet showed no detectable serum cross-reactivity to human ferritin protein in assays.

To get rid of exogenous scaffold dependency entails adequate interactions among hydrophobic core patches for trimerization. GCN4 and its associated disulfide bond in the miniH1 trimer exerted potent homotrimerization, which is thought responsible for base splaying. This led us to consider preserving the original C-helix and gradually introducing interactions across the protomer interface; this gentle approach prevents unexpected deformation of inter-protomer architecture. With a clear guiding ideology that leave what isn't broken, we eventually engineered a scaffold-free H1 stem trimer through structural analysis and iterative designs, designated as #219, which retains native antigenicity (Fig. 1D) (Liu et al., 2024a). Antigenicity assessment demonstrated well-preserved native antigenicity, as indicated by high-avidity binding of #219 to five bnAbs recognizing intra- and inter-protomer B-cell epitopes, respectively, that distribute throughout almost entire stem region. Cryo-electron microscopy solved a high-resolution structural model at 2.97 Å (PDB ID: 9J5L), revealing a nearly identical conformation as the native. Remarkably, a single dose of lipid nanoparticle-encapsulated mRNA expressing #219 protected all mice from fatality after challenge with lethal doses of H1N1 and H5N1. Current design work is to pursue the ultimate in structural precision, yet the breadth of protection still requires further investigation to validate. Several key scientific questions have been raised, whether booster doses can dramatically boost the stem-specific antibody response in clinical trials? How does immunological imprinting by heterologous HA stem epitopes affect the efficacy of #219-based vaccines?

Beyond the stem region, additional dispersed, conserved epitopes include those in the receptor-binding site (RBS), lateral patch and cryptic interface regions of the HA head, and these immunosubdominant epitopes also exhibit cross-protective potential (Raymond et al., 2018; Watanabe et al., 2019; Wu and Gao, 2019; Wu and Wilson, 2020; Matthys et al., 2025). There are already technically feasible approaches for engineering the optimal presentation of individual conformational epitopes consisting of discrete peptide fragments. Notably, computational *de novo* protein design techniques have advanced to offer promising solutions for scaffold development, as exemplified by the work of Fabian (Sesterhenn et al., 2020). Their team achieved success in constructing a novel scaffold that accurately

presents native B-cell epitopes from the RSV fusion (F) protein antigen. They used TopoBuilder to generate *de novo* protein topologies, which specifically accommodate complex functional motifs, this was followed by Rosetta-based *in silico* folding and sequence optimization. A resulting computationally designed antigen presenting a cocktail of native epitopes from F protein elicited balanced neutralizing antibody responses that target each individual epitope and provided immuno-protection in both mouse and nonhuman primate models. This innovative approach breaks through longstanding technical limitations, enabling the rational design of antigens capable of precisely presenting single conformational epitopes.

THE LONG AND WINDING ROAD OF RSV IMMUNOPROPHYLAXIS

RSV is a leading global etiology of acute lower respiratory infections (Li, Y. et al., 2022; Ren and Li, 2024), designated for its characteristic syncytia formation through fusion of infected respiratory epithelial cells into multinucleated giant cells. The virus is pleomorphism, predominantly filamentous at approximately 50 nm diameter and 1–10 μm length (Ke et al., 2018), with spherical particles of 150–250 nm also observed. Classified in thegenus *Orthopneumovirus*, family *Pneumoviridae*, RSV has a nonsegmented negative-sense single-stranded RNA genome encoding 11 proteins and two major serotypes, A and B (Thornhill and Verhoeven, 2020). RSV outbreaks typically peak in winter months, with maximal incidence in December and January. Infants aged 6–12 months are the most susceptible group (Bont and Houben, 2011). RSV accounts for an estimated 60,000 annual deaths in hospitalized children under five (Shi et al., 2017), and substantial excess mortality in the elderly, comparable to seasonal influenza (Schubert et al., 2021; Li, Y. et al., 2022; Progress at last against RSV, 2023; Chaumont et al., 2025). The COVID-19 pandemic perturbed respiratory virus epidemiology via reduced population exposure, while post-pandemic waning immunity has led to notable RSV and influenza resurgences in some regions, overwhelming healthcare systems (Billard et al., 2022; Bardsley et al., 2023; Akhtar et al., 2025; Peng et al., 2025a).

Prior to 2023, no licensed preventive vaccines were available for the prevention of RSV infection. The development of a FI-RSV vaccine nearly six decades ago resulted in a tragic failure (Kapikian et al., 1969). While the vaccine stimulated detectable levels of neutralizing antibodies, vaccinations not only failed to confer RSV protection but also induced ERD upon exposure to natural RSV infection; this ERD subsequently progressed to severe pneumonia following infection, resulting in the deaths of two vaccinated infants. This outcome underscored considerable gaps in the understanding of the molecular mechanism of RSV immunopathology. Importantly, neither natural reinfection nor live-attenuated vaccination causes ERD (Belshe et al., 1982; Wright et al., 2007), and no cases of FI-RSV-associated ERD have been reported in individuals with prior RSV infection, suggesting that immunological imprinting by RSV infection mitigates aberrant responses or shapes protective responses. Subsequently, it was reported that prior RSV infection suppresses vaccine-induced imbalanced Th2-skewed T cell responses that are likely associated with severe immunopathology. Controlled studies further indicated that ERD was specific to FI-RSV but not attributable to aluminum adjuvant or cellular impurities alone (De Swart et al., 2002). FI-RSV elicited high titers of binding antibodies yet with poor neutralizing and fusion-inhibiting activity (Murphy et al., 1986; Murphy and Walsh, 1988), implying a failure to preserve authentic protective epitopes. These findings highlight harmful effects of faulty immune imprinting by FI-RSV vaccine and importantly the necessity of mapping all neutralizing epitopes.

Previous research into RSV infection mechanisms has identified the attachment glycoprotein G and the F protein as key antigenic targets (Mclellan et al., 2013c). The F protein, in particular, has gained central focus due to its essential role in viral entry and relatively higher sequence conservation rate (Mas et al., 2018; Fu et al., 2024). Studies revealed that formaldehyde fixative inactivation predominantly stabilizes the post-F protein on viral surfaces (Killikelly et al., 2016; Zhang et al., 2019), whereas infectious RSV virions present both prefusion and postfusion forms. Many animal studies suggested that immune responses to G and post-F protein antigens might be associated with ERD (Johnson et al., 1998; Schneider-Ohrum et al., 2017; Chen et al., 2023; Kawahara et al., 2024; Luo et al., 2025). Aberrant immune responses implicated in FI-RSV-associated ERD are characterized by inadequate neutralizing antibody production, the formation of non-neutralizing antibody-virus complexes that potentially induce excessive pulmonary complement activation (Polack et al., 2002; Kuppan et al., 2021), as well as dysregulated Th2 inflammation. This Th2 skewing is marked by elevated levels of IL-4, IL-5, IL-13, IgE, histamine, along with mucin hypersecretion, all contributing to aggravated pulmonary immunopathology (Knudson et al., 2015; Ruckwardt et al., 2019; Kawahara et al., 2024). Increasing numbers of isolated potent neutralizing monoclonal antibodies (mAbs) were found targeting epitopes in the pre-F protein, prompting the recognition to prioritize developing pre-F-based vaccine antigens (Mclellan et al., 2013a; Mclellan et al., 2013b; Correia et al., 2014; Mclellan, 2015; Ngwuta et al., 2015; Gilman et al., 2016). This strategy shift to structure-based rational design invariably entails atomic-level structural insights and more well-characterized neutralizing mAbs identifying functional conformation. The world's first well-characterized stable pre-F antigen named as DS-Cav1 was developed in 2013 and showed capabilities to elicit robust neutralizing antibody responses in animal models and humans (Mclellan et al., 2013a; Chang et al., 2022).

Infants up to 24 months of age are most vulnerable to severe RSV diseases. However, no RSV vaccines are currently approved for this age group, likely due to their underdeveloped immune systems, interference from passively acquired maternal antibodies, and stricter safety standards (Graham et al., 2015). By the latter half of 2023, two novel passive immunization regimens were approved by the FDA for infant protection (Trusinska et al., 2025). Maternal vaccination with the unadjuvanted RSVpreF vaccine (Abrysvo, Pfizer) enhances maternal antibody levels that are transferred transplacentally to protect infants in early life. This approach minimizes the risk of ERD, particularly since most pregnant women have preexisting immunity conferred by RSV infections. Vaccination during 32–36 weeks of gestation optimizes antibody transfer, as disclosed in clinical trials, providing up to 81.8% effectiveness against severe RSV in infants under 6 months of age. Nirsevimab (Zhu et al., 2017; Hammitt et al., 2022; Keam, 2023; Ares-Gómez et al., 2024; Chauvel et al., 2024; Sumsuzzman et al., 2025; Wang et al., 2025), a long-acting recombinant human IgG1 kappa mAb developed by Sanofi and AstraZeneca, demonstrated efficacy over a typical RSV season. Crystallographic analysis revealed that this antibody binds the F1 and F2 subunits in the prefusion state at a highly conserved epitope to lock the prefusion conformation. It shows over 50-fold greater *in vitro* neutralizing activity and 9-fold more potent at reducing pulmonary viral loads than palivizumab, the first FDA-approved RSV prophylactic antibody from 1998 (Zhu et al., 2017).

Additionally, three licensed pre-F protein-based vaccines, Arexvy (GSK, AS01-adjuvanted), Abrysvo, and mRESVIA (Moderna), have demonstrated efficacy in older adults, with protection exceeding 90% (Beran et al., 2018; Schwarz et al., 2019; Kampmann et al., 2023; Leroux-Roels et al., 2023, 2024; Melgar et al., 2023; Baker et al., 2024; Tartof et al., 2024; Du et al., 2025; Tanriover et al., 2025). Although specific immunity wanes over time, a single dose of RSVPreF3 OA vaccine maintained 62.9% efficacy against lower respiratory tract disease over three RSV seasons in adults aged 60 or older (Ison et al., 2025), performance comparable to two-dose regimens. Yet this clinical result may cast doubt on the effectiveness and value of booster immunization. On balance, critical advances represent a transformative milestone in the pursuit of safe and effective RSV immunoprophylaxis across diverse age groups.

ENGINEERING APPROACHES FOR RSV PRE-F PROTEIN STABILIZATION

To better illustrate and explain various approaches for vaccine designs, we herein review the structural characteristics of F protein and critical antigenic epitopes. The viral F protein is activated through proteolytic cleavage by furin-like proteases, resulting in the formation of the F1 and F2 subunits, along with the release of peptide 27 (Fig. 2A). This process yields a membrane-anchored homotrimer composed of F1/F2 heterodimers. Similar to other class I viral fusion proteins, the RSV F protein also undergoes extensive conformational rearrangements to mediate viral-cellular membrane fusion, irreversibly transitioning from a metastable prefusion state to a stable postfusion conformation (Fig. 2B). Despite these drastic structural changes, the prefusion and postfusion conformations share approximately 50% of their surface topography (Mclellan et al., 2011). Epitope mapping of anti-F mAbs has identified distinct antigenic sites designated as Ø to V (Fig. 2A) (Kwakkenbos et al., 2010; Corti et al., 2013; Mclellan, 2015; Wen et al., 2017; Battles and Mclellan, 2019; Jones et al., 2019; Ruckwardt et al., 2019; Hammitt et al., 2022; Mccool et al., 2023; Scharffenberger et al., 2024). Complex formation with pre-F-specific neutralizing antibodies stabilizes the pre-F antigen for structural studies, while also providing a critical tool for monitoring pre-F epitope integrity during subsequent antigen engineering (Mclellan et al., 2013a).

Site Ø is the most important pre-F-specific epitope for the elicitation of potent neutralizing antibodies, exhibiting pronounced vulnerability to specific neutralizing antibodies (Magro et al., 2012; Graham et al., 2015). This site is exclusively presented on the membrane-distal apex of the metastable pre-F conformation and is composed of an unstructured region in the F2 subunit (residues 62–70) and the α4-helix within the F1 subunit (residues 196–209) (Fig. 2A), whereas in the postfusion conformation, this site Ø epitope is completely dismantled, with the α4-helix inverting by 180° (Mclellan, 2015). Human-derived potent antibodies D25 and AM22 bind to this site on single protomers of the trimer in different orientations (Kwakkenbos et al., 2010; Mclellan et al., 2013b; Jones et al., 2019). The second pre-F-specific antigenic site is the site V, which is situated immediately beneath and partially overlaps with site Ø. The mAbs targeting site V also provide viral neutralizing activity, such as REGN2222 (suptavumab), CR9501, AM14, RSB1, and MPE8 (Gilman et al., 2019). In contrast, antigenic sites I, II (the binding site of palivizumab), III, and IV (targeted by clesrovimab) are present in both structures of F protein but undergo rearrangement of varying degrees in post-F. For instance, antibodies against site III bind the pre-F with higher affinity, while site I-specific antibodies preferentially target the post-F and are poor neutralizers (Gilman et al., 2016; Peng et al., 2025b). Overall, antibodies targeting sites Ø and V outperform those specific to other sites on neutralization function (Magro et al., 2012). The site Ø, in particular, in turn has fundamentally reshaped the guiding principles of antigen design and is recognized as the holy grail of RSV vaccinology.

Historically, the intrinsic metastability of pre-F protein has long impeded its preparation, hampering high-resolution structural analysis and rational protein design. Early attempts to express the soluble form of the F ectodomain without the transmembrane region in HEp-2 cells failed, as resulting monomers predominantly formed rosette-like aggregates upon cleavage by furin, which essentially lost the native conformation of neutralizing epitopes (González-Reyes et al., 2001; Begoña Ruiz-Argüello et al., 2002; Ruiz-Argüello et al., 2004). Another

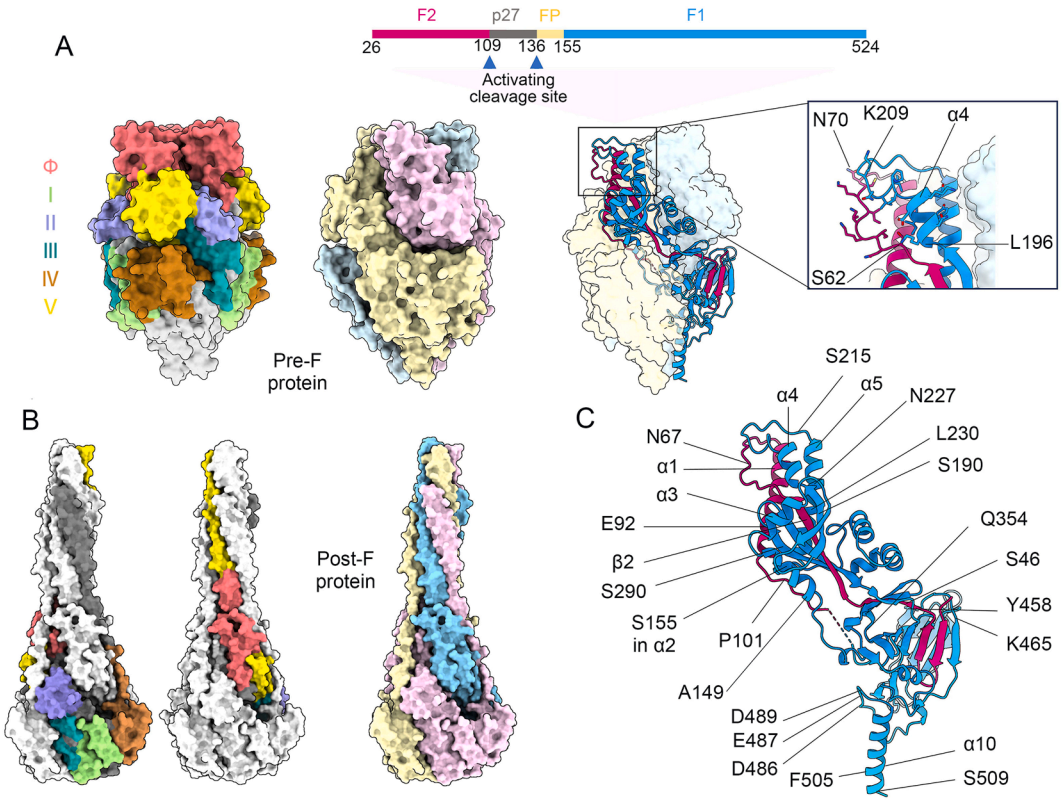

Fig. 2. Schematic diagrams of RSV pre-F and post-F protein antigens and introduction to modification hotspots. **A** Schematic of the trimeric structure of pre-F protein and the localization and distribution of antigenic sites including Ø, I, II, III, IV, and V. The key neutralizing antigenic site Ø is mainly composed of the S62–N70 polypeptide fragment of F2 and the L196-K209 polypeptide fragment of F1. The presented pre-F protein structure model is built based the model with PDB ID: 5C6B. **B** Schematic representation of the post-F protein trimeric structure and the positional distribution of residues of each antigenic site after prominent structural rearrangement. The post-F counterpart is derived from the model with PDB ID: 3RRR. **C** A single F protein protomer (derived from the model with PDB ID: 5C6B) shows multiple critical mutation sites on the structure of stabilized recombinant F proteins and secondary structures emphasized during the modification process.

recombinant variant, F ΔFP (residues 1–513, lacking the fusion peptide at residues 137–146), only formed post-F trimers with an extended, tightly intertwined coiled-coil architecture, as shown by solved crystal structures (Mclellan et al., 2011; Swanson et al., 2011). Jason S. McLellan and colleagues overcame this bottleneck by employing two expression strategies using the HEK293 GnTI$^{-/-}$ cell line, which either co-expressing the light and heavy chains of the D25 Fab antibody alongside a soluble F antigen fused to T4 fibritin trimerization motif, or adding D25 Fab after RSV F transfection (Mclellan et al., 2013b), both generating antibody/pre-F complexes. This work enabled crystallization for X-ray diffraction, and finally yielded a 3.6 Å resolution structure of the pre-F/D25 complex, shedding light onto conformational change mechanisms and laying a solid structural foundation for subsequent rational designs (Mclellan et al., 2013c).

The primary design objective was to engineer a stable pre-F trimer by remedying structural defects so as to retain the antigenic site Ø unique to it. Multiple effective mutation sites for F protein stabilization strategies, as verified by a series of studies below, have been summarized (Fig. 2C). In pre-F, the Cβs of both S155 in α2-helix and S290 in the opposite β-sheet are spatially proximal with 4.4 Å, whereas their distance can reach 124.2 Å in post-F architecture (Mclellan et al., 2013a). Substituting cysteines for this pair of residues created a disulfide bond that covalently tethers two regions, preventing local structural transition (Mclellan et al., 2013a). However, in this disulfide-stabilized (DS) variant, the membrane-distal antigenic site Ø remained disordered, even abolishing D25 binding. Of note, this study found that all intrachain cysteine modifications at regions that do not rearrange between prefusion and postfusion states also did not stabilize the site Ø. Typically, the more tightly compact the core, the less prone to conformational transition. Interprotomer hydrophobic core patches are often suspected of vulnerable hotspots for the stability engineering of class I viral fusion proteins, and cavity filling with suitable hydrophobic residues can serve as a viable approach to lower the free energy of the prefusion architecture. Hydrophobic cavity fillings implemented in the Cav1 construct by substitution S190F increased van der Waals contacts with neighboring residues I57, K176, V192, E256, S259, and L260, while V207L located in site Ø allowed its nonpolar side chain to pack an adjacent hydrophobic pocket tightly (Mclellan et al., 2013a). A shift of Cα by 5.5 Å displacement at the L207 was observed though, compared with the solved structure. An acidic patch located at the membrane-proximal trimer interface of pre-F forms a negatively charged cluster and the consequent charge repulsion therein also drives instability. Therefore, an acid patch neutralization strategy was implemented through substitutions D486H, E487Q, and D489H, resulting in a hydrogen bond between H486 and Q487 as well as hydrophobic stacking between W488 with F140 (Mclellan et al., 2013a). These mutations in conjunction with F488W empowered the TriC variant to trimerize and retain native antigenicity, though with a relatively lower yield. The DS, Cav1, and TriC variants each exhibit improvement, with a notable highlight being the observation from size-exclusion chromatography that Cav1 and TriC undergo a low level of continual conversion from trimer to aggregate. Likewise, this very conversion phenomenon was observed as well in our work on HA stem design, which underscores the necessity of conducting ongoing stability assessments via prolonged storage or freeze-thaw cycles. Combinatorial optimization potentiates synergistic effects. Among over 100 F variants, DS-Cav1 best preserves antigenic site Ø under extreme conditions, such as freeze-thaw cycles, pH 3.5–10.0, 3000 mM high osmolality, and yields 1.9 mg/L exceeding individual variants (Mclellan et al., 2013a). This detailed milestone research work validates the concept for stabilizing pre-F and informs subsequent formulation of design ideas.

Another study engineered a stable pre-F antigen by targeting the structural mechanisms underlying irreversible large-scale rearrangement in refolding regions. Specifically, based on the F protein in which the p27 segment is replaced by a short GS hexapeptide linker (Krarup et al., 2015), proline substitutions are made at four hinge positions to restrict conformational flexibility. The substitution S215P in the α4-α5 loop is particularly effective, resulting in enhanced protein stability and restored nearby conformational epitopes. Proline, due to its constrained backbone dihedral angles, acts like a structural chock that restricts coil-to-helix transition (Hsieh et al., 2020; Olia et al., 2021). Further mutagenesis screens detected the essential stabilizing mutation N67I in the disordered β2-α1 loop with high temperature factor and likely as rearrangement initiation site. N67I strengthens hydrophobic interactions with α1 and α4 helices. Presumably, opting for N67 to mutate, rather than a neighboring residue K65, E66, or K68, is due to its side chain not engaging the D25 paratope, with solved pre-F structures displaying it oriented inward, and as a part of site Ø yet seemingly not involving in hooking cognate receptors. Similarly, charge neutralizing mutations E487Q and D486 N in refolding region 2 eliminated repulsive forces therein, which functions in a manner analogous to TriC. As expected, the top-performing construct SC-DM's superior structural characteristics confer its capacity to induce robust neutralizing protection.

Building on their prior research with the first-generation DS-Cav1, Peter D. Kwong's research team augmented immunogenicity and stability, elevated expression levels, and refined the quaternary structure through iterative structure-based design (Joyce et al., 2016). Additional interprotomer disulfide bonds formed by substitutions A149C/Y458C notably improved trimer stability. In addition to engineering the single-chain 9-10 and incorporating stabilizing mutations N67I and S215P, both previously identified in the SC-DM construct (Krarup et al., 2015), further refinements aimed at resolving specific charge repulsions and steric clashes were achieved via substitutions S46G, E92D, and K465Q. Modifications collectively gave rise to the second-generation DS2 F-glycoprotein antigen that exhibits superior biophysical properties and also enhances RSV protection. The high global stability conferred by a protein variant generally offsets the effects of a local electrostatic repulsive as well as other detrimental interchain interactions in small regions, which are present in the native state simply to accommodate functional necessity. In efforts to engineer improved structural stability, it is necessary to perform in-depth structural analysis for identification and optimization of these minor conflicting areas.

A recent study puts forward an innovative alternative strategy for stabilizing the metastable pre-F conformation via structural and computational analyses, distinguished by grasping dynamic coupling between local structural elements (Liang et al., 2024). Dynamic correlations across protein regions were evaluated using two complementary elastic network models, the Gaussian network model and the anisotropic network model. In conjunction with calculation of entropy transfer across regions, it was delineated that the apex of refolding region 1 exhibits intrinsic large-amplitude motions. Furthermore, such motions were found to drive coordinated bending and shearing movements which occur in between both α1/α5 and these two helices/refolding region 1, which are seen as kinetically coupling correlations (Liang et al., 2024). This finding inspires novel engineering hotspots, instructing on the subsequent engineering direction. As revealed, substitutions N227 M and L230F in α5 strengthened hydrophobic contacts with neighboring residues F223, L230, and Y86, inhibiting helix bending, and enhanced interactions with Y86 and V90 in α1, blocking shearing motions, respectively. DM-9 embracing M227 and F230 showed strong antigenicity, albeit in a metastable state. The acidic patch depicted earlier still merits modification. Rather than the described acid patch neutralization strategy, this study takes an alternative approach by enhancing hydrophobic interactions at the trimer interface to counteract charge repulsion. It was demonstrated that TriM-5 enhanced trimer stability and provided neutralizing protection in vaccinated mice and cotton rats, with efficacy non-inferior to DS-CAV1 (Liang et al., 2024).

We believe that integrating point mutations for interprotomer stabilization at hotspots identified in previous studies holds promise for

eliminating the dependence on exogenous trimerization motifs. A recent critical study successfully engineered a pre-F antigen lacking exogenous motifs. The activation mechanism of RSV F protein via two-site cleavage for the p27 fragment release delays trimerization, resulting in higher intrinsic instability relative to other class I fusion proteins (Bakkers et al., 2024a). This study reveals that foldon-mediated forced trimerization aggravates this instability and leads to rapid refolding to the post-F conformation, only addressing trimer formation rather than inherent structural instability. Molecular dynamics simulations clarify that trimerization-induced instability mainly stems from two regions, destabilization concentrated around the ring of negative charge repulsion at the head-stem nexus near the three-fold axis, involving residues D486 and E487 as previously reported, and a positively charged instability layer within the core, where R106 and R339 from the adjacent protomer interface form a positive repulsive cluster (Bakkers et al., 2024a). Additional instability hotspots in foldon-dependent trimers are situated in the fusion peptide and the 354–357 loop, both of them with high B-factors and functionally required to dissociate during the conformational transition to the post-F state, as well as in the F2 C-terminal region, which is associated with fusion peptide instability. The substitution Q354L decreases the mobility of the 354–357 loop in the central cavity, while D486 N, E487L, and D489Y eliminate repulsion hotspots in the core structure; furthermore, the S215P identified in the SC-TM construct, which stabilizes the hinge loop, and another previously identified substitution P101Q were included in the foldon-free protein mutants (Krarup et al., 2015; Langedijk et al., 2022). Based on a reasonable hypothesis that the HR2 'inverted pyramid' architecture of the upper stem likely continues into a more classical coiled-coil structure in the lower stem, the triple helix of HR2 of the foldon-free pre-F variant was extended by adding 11 additional C-terminal native residues. Molecular characterization experiments confirmed that this protein variant (residues 1–524) exhibited higher yield, increased thermostability, and reduced trimer-to-monomer conversion compared to F variants with a short stem (residues 1–513), furthermore, hydrophobic filling through F505W and S509F substitutions in the three-helix bundle stem region increases trimerization propensity (Bakkers et al., 2024a). The structure of the best-performing RSV-A preF-Δfoldon construct, was resolved up to residue S521 by cryo-EM, which retains the native prefusion conformation, making it the most complete F antigen structure available to date. In this stabilized structure, the aliphatic side chain of R339 interacts with the phenyl ring of F137, while R106 is no longer visible. Animal immunization experiments demonstrated that the preF-Δfoldon protein conferred immuno-protection against RSV infections at both upper and lower respiratory tracts (Bakkers et al., 2024a). The authors proposed that the limited increase in neutralizing antibody titers after receiving boosters of licensed vaccines might be related to foldon competing for immune response resources, offering a new insight into vaccination outcomes. However, whether this will substantially improve booster immunization efficacy as hypothesized awaits clinical trial verification. This engineering strategy for stability increase devised in this study could be explored for application to other viral F proteins, such as those from the parainfluenza virus and human metapneumovirus.

The elicitation of site Ø constitutes a substantial portion of the human neutralizing responses to natural RSV infection, spurring immuno-focusing designs. The membrane-distal head domain of intact pre-F where the site Ø resides, consisting of F2 residues 51–104 and F1 residues 146–306, was engineered into a stable head-only antigen via linkers and appending a coiled coil trimerization motif at the N-terminus (Boyington et al., 2016). Vaccinations with this antigen induced RSV neutralizing immunity comparable to the parental DS-Cav1. Additionally, enzymatic removal of the foldon dissociates F protein into monomers. With the aim of removing the phage foldon while stabilizing pre-F protein, cysteine residues were introduced into the C-terminal coiled coil hinge region of F ectodomain (Stewart-Jones et al., 2015), forming stable interprotomer disulfide bonds.

CONCLUDING REMARKS

In recent years, a variety of novel broadly protective influenza antigens and potent RSV F antigens have been reported. Of note, vaccine design strategies and objectives for these two pathogens influenza and RSV are distinct, while remaining highly representative for current antigen design efforts against viral pathogens. Engineering goals, such as the independent HA stem domain and the RSV pre-F conformation, were determined by high-resolution mapping of identified functional epitopes. Nevertheless, stabilizing mechanisms utilized in these studies, including the establishment of interprotomer disulfide bridge, hydrophobic packing, and proline substitution, were originally validated during quests for effective HIV vaccines. Currently, the theoretical framework of structural vaccinology is evolving at an unprecedented depth, integrating technical expertise from vaccine immunology, immune receptor omics, vaccine genetics, physical modeling, and artificial intelligence, so as to tackle tough challenges in the design of either universal or even just effective vaccines against pathogens, like HIV and HCV. Due to the high mutation frequency of HIV, the stabilized SOSIP Env antigens remain distant from achieving efficient neutralizing protection, making it necessary to continue exploring precision immune strategies targeting the affinity-maturation trajectory of like VRC01-class bnAbs (Haynes et al., 2023; Gieselmann et al., 2025; Lin et al., 2025). Sequential vaccination with germline-targeting immunogens, for instance, enables the phased activation of the host precursor B cells, which gradually matures the affinity of bnAbs (Haynes et al., 2023). In short, vaccine development has undergone a distinct paradigm shift, moving beyond producing vaccines based on natural viral antigens toward innovative vaccine antigens tailored to targeted or predefined immune responses. Reverse vaccinology 2.0 is propelling this shift into an era of more rational, efficient, and precise strategies, with a kind of universal vaccine probably to be the next disruptive achievement in the foreseeable future.

Analyzing the conformational dynamics of metastable class I fusion proteins during prefusion to postfusion unfolding is crucial for identifying potential mutation sites for stabilization especially the structurally vulnerable initiation points in the major conformational transition pathways involving intermediates (Banerjee et al., 2015). This has been well-illustrated by the RSV pre-F antigen design process and has also facilitated the stability engineering of both SARS-CoV-2 S–2P and HexaPro (Hsieh et al., 2020). Near-atomic visualization of morphologically distinct metastable refolding intermediates is essential for elucidating conformational transitions and probably provides mechanistic insights to guide stability designs. Single-molecule experiments can be accomplished using alternating laser excitation in conjunction with single-molecule Förster resonance energy transfer (Impagliazzo et al., 2015), a technology that resolves distinct molecular states and their dynamics under equilibrium or non-equilibrium conditions. Cryo-electron microscopy, cryo-electron tomography, and hydrogen/deuterium exchange mass spectrometry have emerged as indispensable tools for characterizing class I fusion protein intermediate structures (Li et al., 2016; Riedel, et al., 2017; Benhaim et al., 2020; Benton et al., 2020; Gao et al., 2020; Marcink et al., 2020; Ward et al., 2020; Marcink et al., 2022; Li et al., 2023; Song et al., 2023), with well-documented applications for viral proteins such as SARS-CoV-2 spike protein, Lassa arenavirus glycoprotein, human parainfluenza virus fusion protein, HIV Env, murine leukemia virus Env, influenza virus HA, and so on. Molecular dynamics simulations offer further atomistic insights through comprehensive computational analysis of transition pathways. Generative adversarial networks sift critical intermediate structures and characterize simulated trajectories using extensive molecular dynamics data. In addition, strategic stabilizing mutations typically do not overlap with identified B-cell epitopes and when trade-offs are needed sites are prioritized based on balancing residue conservation and epitope engagement. Overall, insights gained from cutting-edge structure-informed vaccine research are widely

applicable to antigens derived from other pathogens. It is worth noting that while optimal antigen design is a fundamental step in vaccine development pipeline, scientific gaps still necessitate further clinical research efforts to address. Factors such as maternal antibody interference in infants, heterologous immune imprinting across diverse populations, immune receptor omics profiles, and the applied vaccine vectors and adjuvants, all shape immune response profiles.

Beyond traditional physical modeling, structural expertise and intuition as well, generative artificial intelligence models also support reverse vaccinology research. Web-based Vaxign-ML platform leverages machine learning trained on amino acid residue chemical properties of known pathogenic protein antigens, delivering robust protective antigen predictions (Ong and He, 2022). Experimentally validated PLGDL framework synergizes protein language and geometric deep learning, enable high-performance proteome-wide screening for protective antigens (Zai et al., 2025). DeepMind's AlphaFold addresses high-quality protein folding and stability prediction, making rational protein design without experimental structures possible (Chica and Ferruz, 2024). Advanced deep learning models now tackle increasingly sophisticated design needs, such as B-cell epitope prediction, mapping footprint (Ivanisenko et al., 2024; Gabellieri et al., 2026) and T-cell epitope immunogenicity prediction (Wohlwend et al., 2025). Arguably, the diffusion model-driven *de novo* protein design approach, which can also be applied restrictedly for local modifications of a defined subdomain via semi-rational approach, holds potential for engineering protein structure and functionality, representing a valuable direction for future integration and exploration. Collectively, reliable artificial intelligence tools complement and advance cutting-edge vaccine development system.

Integrating human immunology with structure-based design of novel antigens is a prominent trend for developing next-generation vaccines. This review has a limitation in that not all thought-provoking proof-of-concept studies on structural vaccinology regarding novel influenza and RSV vaccines are included, due to our limited knowledge reserve and the article length. This article focuses on structural stabilization mechanisms; nevertheless, designing more novel and effective vaccination strategies necessitates further in-depth investigations into immune molecular aspects on this foundation, such as the generation trajectories of broadly neutralizing antibodies, T-cell receptors, and the exploitation of emerging artificial intelligence technologies. All of these aspects denote the complexity of design conception and strategic evaluation of novel vaccines but are not reviewed in this article.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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