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Protein engineering and particulate display of B-cell epitopes to facilitate development of novel vaccines

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Induction of antigen-specific humoral immunity is a correlate of protection for many diseases and remains a primary vaccine goal. Pathogens can evade such responses by limiting epitope access, by diversifying surface residues, or by keeping antigens in metastable conformations. B cells can target diverse epitopes on an antigen, but only a subset of which produce functional antibodies. Structure-based immunogen engineering can help overcome these hurdles by using structural information for targeted induction of particular antibodies while improving the overall vaccine immunogenicity. This review will cover recent progress in vaccine design, specifically focusing on strategies to stabilize antigens for optimal B-cell epitope exposure, engineer synthetic B-cell epitopes to induce antibodies with specific features and enhancement of vaccine potency through antigen presentation on multivalent particles.

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Introduction

Vaccination has been one of the most important public health measures in history. However, there are still significant limitations in the coverage of current vaccines and 19.3% of deaths in 2016 were caused by communicable diseases [1]. Adoption of new strategies for vaccine development is, therefore, of significant importance to global health. Vaccines may mediate protection through multiple redundant and synergistic mechanisms, with the induction of specific antibodies being highly valued as a correlate of protection in most vaccines [2]. A primary goal of vaccination, therefore, is to induce humoral memory by creation of antigen-specific, long-lived plasma cells and antigen-experienced memory B cells. Induction of tailored humoral immunity requires the use of antigens with precise molecular surfaces with particular physiochemical properties, inspiring the field of structural vaccinology (SV), which is the subject of this review. SV is a powerful tool where concepts in protein engineering are applied to design new vaccine immunogens and improve upon existing vaccine immunogens.

In the context of SV, approaches in rational protein engineering can be characterized as: 1) structure-guided design (SGD), 2) computational protein design (CPD) or 3) directed evolution (DE). SGD is the most straightforward technique where mutations to vaccine immunogens are selected by visual inspection of existing protein structures. CPD uses computer algorithms which can explore massive sequence spaces, model drastic structural changes, and discover correlated mutations. SGD and CPD are dependent on the existence of structural information. The DE approach is not strictly dependent on structures; instead libraries of variants are screened against neutralizing antibodies (nAbs), non-neutralizing antibodies (non-nAbs), soluble cell surface receptors, or ligands. Maturation of immunogens is often achieved through iterative cycles where combinations of mutations are introduced by random, structure-directed or saturated mutagenesis methods. DE is ideally suited to evolve immunogens when both a well-folded starting structure exists, and the target antibody has detectable affinity ($<50\,\mu M$). This review will cover several novel vaccine candidates that were engineered through SGD, CPD and/or DE.

Harnessing structural information for vaccine design

In the past decade, structure-based vaccine design efforts have been dramatically accelerated by the increased availability of new viral glycoprotein and antibody structures. For example, a recent high-resolution Cryo-EM structure of the wild-type membrane-bound HIV-1 envelope (Env) protein has helped define the molecular architecture of this important vaccine target in its native environment [3]. Additional Cryo-EM structures of Env trimer in complex with its cell surface receptor, CD4, highlight conformational changes that occur upon receptor engagement [4,5]. Further, structural insights into glycan processing of viral glycoproteins are helping us understand how viruses evade host immunity and elucidate strategies to circumvent such challenges [6–8].

The discovery of functional antibodies yields insights for the design of new vaccines. Structures of neutralizing

antibodies in complex with viral surface proteins provide a structural rationale for antibody-mediated neutralization, helping identify critical B-cell epitopes for targeted vaccine efforts [3,6,9–13]. In particular, humoral responses can now be quickly assessed by obtaining structural information on elicited antibodies. Dingens *et al.* recently used Cryo-EM to study the differences in antibodies that arose during natural infection from those elicited upon vaccination, illustrating a plausible pipeline to evaluate whether designed antigens can elicit relevant responses [14]. In addition, vaccine-induced humoral responses can be rapidly interrogated by utilizing 3D negative-stain electron-microscopy reconstructions to provide a B-cell epitope blueprint for designed immunogens [15••].

Given the important role of antibodies in informing vaccine design, serological samples are routinely queried by techniques including: single B-cell cloning, memory B-cell immortalization, B-cell culturing and *in vitro* antibody selection systems [16]. The retrieval of antigen-specific antibody sequences from infected or vaccinated hosts is largely predicated upon the probes used during the sorting process. Rational design of the probes with a structure-based approach can result in discoveries of novel antibodies. For instance, development of CD4-binding site knock-out HIV gp120 protein and stabilized native-like trimers enabled the discovery of the broadly neutralizing antibodies (bnAbs) VRC01 [17] and PGDM1400 [18], respectively. Engineering epitope-knockout probes can also allow us to evaluate generation of epitope-specific antibody responses [19–21]. For antibody-focused vaccine efforts, it may be critical to evaluate the frequency of the corresponding antibody class in the naïve human antibody repertoire [22–25]. Two recent studies extensively explored the human antibody repertoire and described a combined 369 million unique antibody sequences from 16 healthy donors and will serve as valuable reference points for future studies [26••,27••].

Advances in immunogen design

Enhancing presentation of native and designed B-cell epitopes

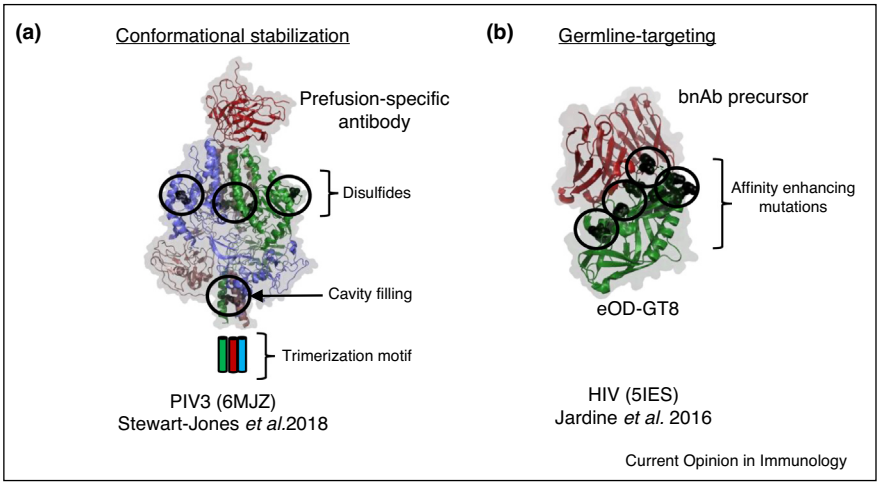
One major focus of immunogen design can be framed as the building of a molecular surface which mimics B-cell epitopes from a pathogen. The molecular surface consists of amino acids, glycans and tightly bound water molecules arranged in a particular geometry that can be recognized by antibodies. Pathogens harbor proteins with conformational metastability at different lifecycle stages which results in the manipulation of their molecular surfaces. Conformational stabilization techniques can be employed to restrict sampling to structural states which contain neutralizing epitopes.

One such example is the stabilization of soluble HIV-1 Env trimers. Although an engineered clade A trimer, BG505.SOSIP.664, has a molecular surface that closely resembles the functional Env spike in terms of its antigenic profile, both nAbs and non-nAbs were induced upon vaccination [28]. Some induced non-nAbs targeted the immunodominant hypervariable V3 loop, which is buried and not accessible to antibodies in the prefusion conformation [29] and yet becomes accessible to B cells *in vivo*. To suppress V3 loop exposure *in vivo* due to CD4 receptor engagement, multiple protein design strategies have been employed. Guenaga *et al.* [30], Kwon *et al.* [31] and Zhang *et al.* [32] added additional disulfide bonds. Liu *et al.* discovered a secondary CD4 contact site amenable to mutagenesis [33]. We recently used multi-state CPD to discover a single disruptive mutation in the CD4 binding site [34]. To limit V3 loop exposure before CD4 receptor engagement, multiple protein design strategies have been explored and demonstrated to improve antigenic profiles and immunogenicity, including addition of new disulfide bonds [35], chemical cross-linking [36], shortening and rigidification of flexible loops [37], novel mutations derived from DE [21] and incorporation of a hydrophobic core at the base of the V3 loop by CPD [34].

In the case of respiratory syncytial virus (RSV) and parainfluenza virus (PIV), the metastable fusion glycoprotein (F) has been identified as a target for protective humoral immunity. McLellan *et al.* reported that a prefusion stabilized F protein can induce high titers of neutralizing antibodies against RSV in mice and macaques [38]. Stewart-Jones *et al.* successfully generalized this result and stabilized human parainfluenza F in the prefusion conformation using SGD techniques [39••]. Guided by pre-fusion structures of simian PIV5-F and post-fusion human PIV3-F, stabilization was achieved by adding a heterologous trimerization domain and by mutating mobile positions between pre-fusion and post-fusion conformations into disulfide bonds and cavity-filling amino acids (Figure 1a). Pre-fusion designs of PIV1–4 F induced higher titers of virus-neutralizing responses in non-human primates (NHPs) than non-stabilized PIV F proteins [39••].

One mechanism that pathogens employ to evade host immunity is sequence diversification, which may be countered through engineering of B-cell epitopes to contain consensus sequences. The elicited immune responses to seasonal multi-valent influenza vaccines are strain-specific, offer minimal cross-protection to strains not included in the regimen and can result in low vaccine effectiveness [40]. To address this issue, Yan *et al.* identified four HA sequence clusters from an influenza H1-subtype phylogenetic tree and utilized them to develop four micro-consensus HA sequences to cover the sequence diversity within each cluster [41]. The vaccine induced protective titers of antibodies against unmatched H1-subtyped viruses that span the past 25 years of seasonal H1 strains in mice, guinea pigs and non-human primates. Elliott *et al.* pursued a consensus approach for

Figure 1



Engineering enhanced B cell epitopes.
(a) An example of conformational stabilization showing the structure of the designed parainfluenza virus 3 F-protein bound to a prefusion-specific antibody. **(b)** An example of germline-targeting design, in which the germline-targeting immunogen, eOD-GT8, is in complex with a VRC01-class precursor antibody. Modifications in antigens for conformational stabilization or affinity enhancement are circled and labeled. The virus, structural identifiers and citation references are annotated below each figure.

H3 influenza strains, designing consensus vaccines capable of inducing high titers of antibodies and potent T-cell responses. Even though not all H3-subtypes were neutralized by vaccine-induced antibodies, the vaccine offered complete protection to mice from two unmatched influenza H3 challenge strains, showing the induced humoral and cellular immunity may work in synergy [42].

With the discovery of many new bnAbs, there are significant efforts to re-elicit similar lineages of antibodies by vaccination. For HIV, it has been observed that unaltered antigens have so far failed to induce bnAbs, possibly due to an inability to activate the precursor B cells of the targeted bnAb. One new vaccine strategy called germline-targeting (GT), aims to prime and boost precursor B cells toward B cells producing antibodies with bnAb-like functionality. GT immunogens are generally constructed in two different ways. Epitopes from pathogens found in individuals who induced bnAbs can be mimicked to create a GT immunogen. Alternatively, GT epitopes can be designed *de novo* to harbor a sequence capable of activating and boosting bnAb precursors. A HIV GT immunogen, engineered outer domain (eOD-GT8), was created using CPD and DE by screening against 18 VRC01-class bnAbs precursors [23] (Figure 1b). eOD-GT8 has high affinity for VRC01-class precursors [23] and induces VRC01-class B cell responses in many knock-in mouse models [20,43,44]. eOD-GT8 was used to isolate naïve VRC01-class B cells from multiple healthy human donors [23,25] and is currently in a phase I safety and immunogenicity trial [45]. Another candidate germline-targeting immunogen for the VRC01-class bnAbs is an optimized gp120-core construct derived from the 426c HIV isolate that can also bind to inferred germlines of the VRC01-class and elicit epitope-specific responses in certain knock-in mice [46]. GT immunogens built from soluble trimeric gp140 variants are also being explored. Steichen *et al.* developed a series of immunogens including a GT prime, 11MutB, which has low micromolar affinities for a panel of PGT121 precursors and can prime mice that harbor PGT121 precursor B cells [21]. When the mice were sequentially boosted with less mutated immunogens, PGT121-like neutralizing antibodies could be elicited [47]. Recently, a gp140 immunogen, BG505 SOSIP.v4.1-GT1, was designed to target both VRC01 and Apex-bnAb class germline precursors, and was found to be capable of inducing epitope-specific responses in a knock-in mouse model harboring VRC01-class precursors, although further work may be required to determine whether the antibody responses arose from conventional murine B cells or VRC01-class precursor B cells [48]. In summary, these studies employ SV approaches for the manipulation of vaccine antigens in order to present desired B cell epitopes through conformational stabilization, consensus engineering, or germline-targeting.

Epitope-focused vaccine design

A second major immunogen design focus is to remove immune distracting B-cell epitopes from an immunogen. Complex multi-epitope antigens contain intrinsic immunodominance hierarchies in which some surface segments may preferentially expand B cells in germinal centers [49–51]. Thus, protein engineers employ diverse epitope-focusing techniques to 'focus' the antibody response onto

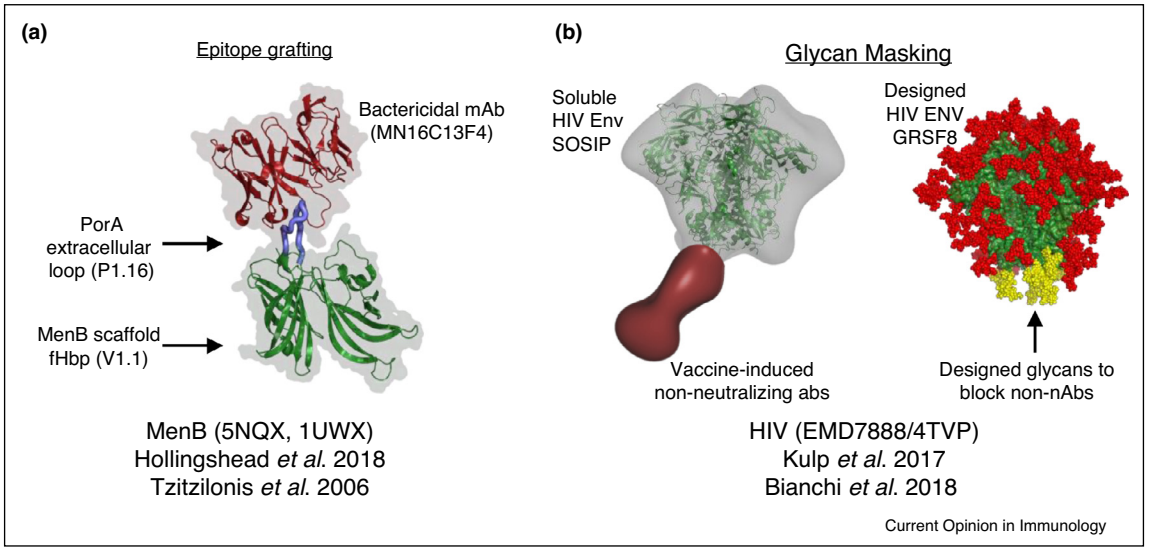
epitopes of interest. These can be grouped into three broad categories. Epitope isolation is the process of grafting a neutralizing epitope onto an unrelated protein scaffold. Domain minimization is the process of reducing a multidomain protein to a small single domain which hosts the neutralizing epitope. Resurfacing is the process of altering distracting epitopes to become subdominant.

In context of a pathogenic bacteria, *Neirsseria menigitidis*, Hollingshead *et al.*, engrafted the VR2 epitope from an integral membrane protein (PorA), which is a target of bactericidal antibodies [52], onto a soluble molecular scaffold protein, fHbp (Figure 2a). The fHbp-VR2 chimeric immunogen successfully elicited antibody responses to both the VR2 epitope and fHbp in mice [53•]. The domain minimization approach was extensively pursued to focus humoral immune responses to the stem domains of HA in an effort to create universal influenza vaccines. Impagliazzo *et al.* [54] and Yassine *et al.* [55] constructed mini-HA stem antigens by removing the immunodominant head domain, introducing stabilizing mutations in the HA stem domain and incorporating the trimerization domains. The domain minimized antigens showed higher protective efficacy in mice relative to full length HA [56] and induced cross-protective responses in influenza-experienced NHPs [57]. Resurfacing is an alternative method to silence the immunodominant HA head domain. In one resurfacing example, chimeric HAs with exotic avian HA head domains and human HA stem domains could be used to develop cross-reactive stem antibodies in heterologous sequential boosting regimes to protect ferrets from influenza challenges [58] and are being evaluated in the clinic [59]. Lastly, immunodominant epitopes can be resurfaced through introduction of N-linked glycans (glycan masking). Duan *et al.* engineered glycans distal to the CD4 binding site on eOD-GT8 to improve the recovery of CD4 binding site-specific antibodies upon vaccination [60]. Engineered glycans on the exposed base of soluble HIV trimer immunogens reduced binding of vaccine-induced, base-directed non-neutralizing antibodies [34] (Figure 2b). To sum up, epitope isolation, domain minimization, and resurfacing may be used alone or in combination to focus responses to segments of antigenic surface crucial for antibody-mediated neutralization.

Engineering multivalent antigen display

Enhancing vaccine efficacy through multivalent antigen display is an approach that has attracted significant interest. Naturally occurring non-viral protein cages, *de novo* designed protein shells and coiled-coil assemblies can each be reengineered to display vaccine antigens. These approaches have demonstrated remarkable utility in enhancing vaccine potency (Table 1). Tokatlian *et al.* recently unveiled a plausible immunological mechanism of action for these nanoparticulate vaccines, highlighting how activation of the innate lectin-complement pathway by nanoparticles might contribute to their rapid trafficking to the draining lymph node for the induction of immune responses [61•].

Figure 2



Epitope-focused immunogen design.
(a) An example of epitope grafting in which an extracellular loop (blue), targeted by a bactericidal antibody (red), is transferred onto the MenB scaffold fHbp protein (green). **(b)** An example of glycan masking, which shows vaccine-induced non-neutralizing antibody (red surface) binding to the bottom of the HIV Env trimer (white surface, green cartoon) and a new designed trimer, GRSF8 (green surface with glycans as red spheres), with incorporation of new glycans (yellow spheres) at the base restrict access of antibodies to such epitopes. The virus, structural identifiers and citation references are annotated below each figure.

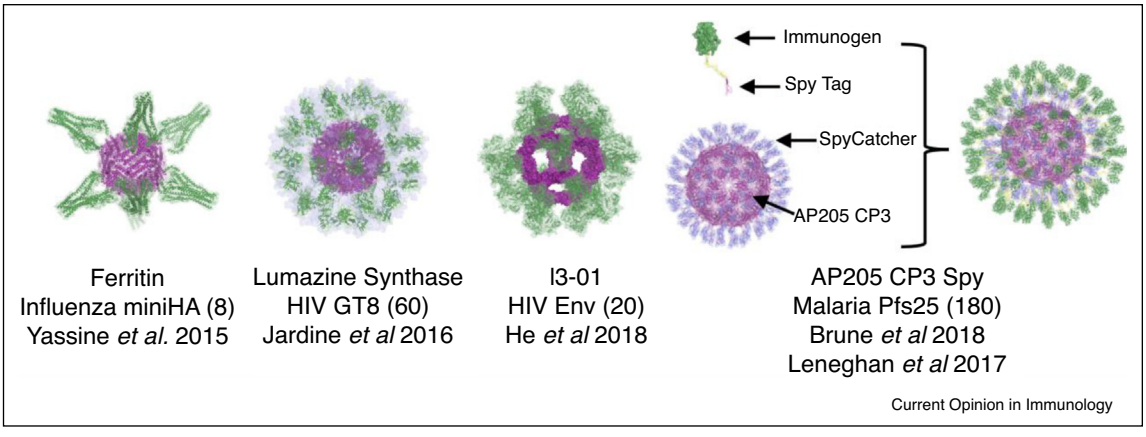
Table 1

Summary of nanoparticle scaffolds used to present immunogens

Nanoparticle	Source	Shape	Valency	Size (nm)	Decorated antigens
Ferritin	*Helicobacter pylori*	Octahedral	8/24	12	HA stem [55] HA RBD [66••] HIV BG505.MD39 [61•]
Lumazine synthase	*Aquifex Aeolicus*	Icosahedral	60	30	HIV eOD-GT8 [23]
E2p	*G. stearothermophilus*	Icosahedral	20	23	HIV BG505.gp140 [67]
I3-01	*De novo* design	Icosahedral	20	25	HIV BG505.gp140 [68]
Encapsulin	*T. maritima*	Icosahedral	60	24	EBV gp350.D_{123} [69]
Major vault protein	eukaryotes	Barrel-shaped	78	40 × 67	HIV Gag [70]
Qβ/AP205 coat proteins	bacteriophage	Icosahedral	180	26	Malaria Pfs25 [71,72•]
Coiled-coil-based cages	*De novo* design	Octahedral	24	20	HA Helix C/M2e [73]

Several recent studies reported that ferritin nanoparticles decorated with engineered influenza HA [55,62] or HIV Env [61•,63,64] elicit stronger humoral responses than corresponding monomers (Figure 3). Georgiev *et al.* recently demonstrated that two-component ferritin nanoparticles composed of both HA and HIV antigens could induce neutralizing antibodies to both viruses, demonstrating the feasibility for using nanoparticle vaccine cocktails [65]. Kanekiyo *et al.* created 'mosaic' ferritins from HA receptor-binding domains of eight influenza isolates and reported that the mosaic nanoparticles induce broader responses than either a mixture of eight individual ferritin nanoparticles or equivalent sequential immunizations [66••]. The potency of these nanoparticle vaccines may be further enhanced. While the ferritin scaffold is capable of displaying 8 copies of a trimeric immunogen or 24 copies of a monomeric immunogen, 60-subunit icosahedral protein assemblies can display 20 trimers or 60 monomers. For example, native-like HIV-1 Env trimers have been engineered to be displayed on E2p [67] and I3-01 [68] scaffolds. Lumazine synthase has been decorated with 60 eOD-GT8 monomers [19,24] (Figure 3). The multivalency of GT-60mers is important for priming broadly neutralizing antibody responses in knock-in mice [19] and activating rare bnAb precursors [24]. Encapsulin is a 60-mer scaffold that has been used to display Epstein–Barr Virus antigens [69]. Coiled-coil driven assemblies, where homotrimeric and heterodimeric coiled-coils are fused together, were used to present two influenza epitopes (the ectodomain of the M2 protein and helix C of the HA protein) along with a TLR5 agonist (flagellin) to induce high titers of antibodies against both epitopes [73]. Recent structures of an engineered major vault protein in fusion with HIV Gag consist of 78 subunits may pave the way for using this nanoparticle in future vaccine studies [70]. For genetic-fusion-based nanoparticles, proper assembly requires design and testing of optimal nanoparticle-linker-immunogen fusions, which is

Figure 3



Engineering multivalent antigen display.
Four examples of self-assembling nanoparticles decorated with various immunogens. From left to right, Ferritin is decorated with 8 stem-only HA trimers, Lumazine Synthase is decorated with 60 copies of eOD-GT8s, I3-01 is decorated with 20 copies of HIV Env trimers and an assembly system, in which an immunogen fused to a SpyTag peptide binds irreversibly to SpyCatcher fused to AP205 CP3 bacteriophage coat becomes decorated with 180 copies of the immunogen. The nanoparticles subunits are shown in deep purple and the immunogens in forest green. The nanoparticle scaffold, the fused antigens, particle valency and citation references are annotated below each figure.

a time-consuming process. To address this pitfall, an innovative 'plug-and-play' vaccine nanoparticle platform was recently developed, using the SpyTag-SpyCatcher system to display any immunogen of interest in a two-component system [72•] (Figure 3). Overall, using nanoparticles to scaffold and present immunogens has been demonstrated as a viable strategy for the induction of more potent functional humoral immunity.

Concluding remarks

Protein engineering techniques can address a vast and diverse set of vaccine development impediments and can be employed to build novel and more potent immunogens. In the past decade, improvements in antibody sequencing and structural determination techniques have significantly advanced structural vaccinology studies and will continue to shorten the cycles of vaccine design and animal experimentation. This review highlights several techniques in SV, such as conformational stabilization, epitope focusing, and multivalent antigen display, which have resulted in designs of several new promising vaccine candidates. Some of the key issues that remain are ways of improving *in vivo* structural stability of designed immunogens, evaluating antigen molecular surfaces decorated on the nanoparticles, and elucidating the key immunological mechanisms that underlie various nanoparticle platforms and vaccine delivery systems. In each case, innovative protein engineering methodologies have and will continue to play an important role in the design of future vaccines.

Conflict of interest statement

Z.X. and D.W.K. have a pending patent US.62784318.

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