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Functional assessment and structural basis of antibody binding to human papillomavirus capsid

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Running Head: Functional epitopes on human papillomavirus capsid

Keywords: human papillomavirus, pseudovirions, neutralizing antibodies, functional epitope, flexible loops, viral capsid

Abbreviations

HPV, human papillomavirus; BPV, bovine papillomavirus; VLP, virus-like particle; Ab, antibody; Ag, antigen; PsV, pseudovirion; HSPGs, heparin sulfate proteoglycans; cryoEM, electron cryomicroscopy; Fab, antigen-binding fragment; SEAP, secreted alkaline phosphatase; cLIA, competitive Luminex immunoassay.

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Summary

Persistent high-risk human papillomavirus (HPV) infection is linked to cervical cancer. Two prophylactic virus-like particle (VLP)-based vaccines have been marketed globally. Here, we review the approaches employed to generate HPV pseudovirions (PsV) that mimic native virions and to assess the neutralization activity of HPV antibodies in patient sera. The PsV-based neutralization assay was developed to study the virology of HPV and to evaluate vaccine efficacy. Specifically, this system has been used to evaluate the efficacy of neutralization antibodies in sera elicited by vaccination or natural infection or to assess the functional characteristics of monoclonal antibodies. We also review the antibody binding modes observed in virus-antibody complexes from the work done on virions, PsVs or VLPs. The neutralizing epitopes are localized on surface loops of the L1 capsid protein, at various locations on the capsomere. Different neutralization antibodies exert their neutralizing function via different mechanisms. Some antibodies neutralize the virion by inducing conformational changes in the viral capsid, which can result in concealing the binding site for a cellular receptor like 1A1D-2 against dengue virus, or inducing premature genome release like E18 against EV71 [1, 2]. Higher-resolution details on the epitope composition of HPV neutralizing antibodies would shed light on the structural basis of the highly efficacious vaccines and aid the design of next generation vaccines. For the current vaccines, certain assays can be developed on the basis of epitope information for improved quality analysis and for monitoring the manufacturing process to ensure product consistency. This is critical in

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the comparability exercise for process improvement or process scale up to meet market demand. For next generation vaccine design, better understanding of the structural basis of the type-specific functional epitopes could aid the design of antigens with cross-type protection activity through epitope redesign or recombination such as loop grafting in hybrid VLPs [3-5].

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Introduction

Human papillomavirus (HPV) is recognized as the causative agent of cervical cancer and virtually all cervical cancers are related to the presence of oncogenic HPV DNAs [6-8]. In addition, genital warts are also caused by non-oncogenic HPV types although they are regarded as benign. HPVs are small non-enveloped DNA viruses that belong to the *Papovaviridae* family. Two late viral proteins, the major L1 and the minor L2, form the viral capsid [9, 10]. The L1 protein can spontaneously self-assemble into virus-like particles (VLPs) [11]. Currently, two globally licensed VLP-based vaccines, Gardasil® and Cervarix® (initially introduced in 2006) have been proven to effectively prevent HPV infection and to reduce HPV-related morbidity [12-17]. The two vaccines can induce a strong immune response and elicit functional L1-specific antibodies. The conferred protective immunity is primarily due to the viral neutralization function of the antibodies that may bind the incoming authentic virions[18-21]. As reported in the literature, all the identified antibodies against L2 such as RG1, 14H6, K4L2 andK18L2 were raised using the recombinant L2 protein. The neutralizing activities of these antibodies were demonstrated with the PsV-based neutralization system.[22-24] Because viral neutralization appears to involve both L1 and L2, the pseudotyped virus (PsV) neutralization model was developed to study the virology of HPV and evaluate the vaccine potential of the VLPs. The PsV-based neutralization assay was utilized to evaluate the efficacy of neutralization antibodies in sera elicited by vaccination or natural infection. Different PsV systems encapsidating the SEAP or RFP as the reporter have been used to

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evaluate the neutralization titers in the serum obtained from VLP-vaccinated women, or women naturally infected with HPV, or prenatal pregnant women [25-27]. PsV was generated by transfecting the L1 and L2 genes into the 239FT cells. PsVs encapsidating the reporter gene were harvested by lysing the cells. Subsequently, the antibodies with a serial dilution was mixed with the titered PsV and then the mixture was added into the 239TT cells. Antibody-mediated PsV neutralization is evaluated by detecting the reporter activity such as SEAP activity [28]. Neutralizing antibodies play a key role in preventing viral infection via different mechanisms [29]. The neutralization mechanisms were reflected in the different binding sites located on the major capsid protein L1 or the minor capsid protein L2. If the high-resolution structures of Ab-Ag complexes were determined, a better understanding of the neutralization mechanism of different mAbs would be achieved. However, this information is rather limited at this stage. Such knowledge of the details of identification and characterization of binding sites for neutralization antibodies would aid in the design of the next generation of vaccines and in the quality control of existing vaccines during vaccine manufacturing [30].

Mimicking native virions with HPV pseudovirions

The lack of permissive and productive cell cultures for HPV has impeded the study of virus-neutralizing mechanisms of antibodies, the monitoring of viral infection and other virological studies [31]. With the need for measuring neutralizing antibodies to high-risk genital HPVs such as HPV16 and HPV18 in the evaluation of vaccine

efficacy, pseudovirion expression systems have been developed for the *in vitro* generation of HPV pseudovirions and for a quantitative *in vitro* assay for infectivity evaluation (Figure 1).

In early studies, Zhou *et al*. used recombinant vaccinia virus to produce bovine papillomavirus type-1 (BPV-1) VLPs containing both L1 and L2 capsid proteins *in vitro*. This system served as an *in vitro* model to identify the BPV-1 cellular receptor [32]. In a separate study, Semliki Forest virus was chosen by Roden *et al.* to express the papillomavirus virion proteins for generating pseudotyped papillomaviruses, *in vitro* [33]. This system has not only permitted the analysis of the ability of antibody cross-type neutralization but also provided insight into the mechanism and specificity of papillomavirus genome packaging and infection.

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Unckell *et al*. generated HPV33 pseudovirions by the assembly of VLPs in COS-7 cells containing multiple copies of a marker plasmid [34]. In addition, HPV18 pseudovirions were prepared using recombinant vaccinia viral expression vectors that were transfected into mammalian 293T cells [35]. This type of pseudovirion was shown to be infectious, as it could transfer β-galactosidase activity or confer resistance to puromycin to a number of cell types when the pseudovirions encapsidated plasmids containing either the β-galactosidase gene or the puromycin-resistance gene, respectively. This finding indicated that intracellular episomal DNAs of suitable sizes can be encapsidated by the HPV18 L1 and L2 proteins without the need for any HPV packaging signal, facilitating the infection of other cells. This finding facilitated the development and refinement of subsequent

pseudovirion neutralization systems. Kawana *et al*. developed a cell-free system for generating infectious HPV16 pseudovirions [36]. The HPV16 capsid proteins L1 and L2 were expressed and co-assembled into VLPs in insect cells (Sf9). The HPV16 VLPs were then disassembled in the presence of the reducing agent, 2-mercaptoethanol and reassembled by removal of the reducing agent in the presence of a β-galactosidase expression plasmid. The *in vitro* construction of this pseudovirion system with marker plasmids would be potentially useful in developing an assay for evaluating virus-neutralizing antibodies from animal or human sera. Coursaget and colleagues also used the similar disassembly/assembly method on VLPs isolated from insect cells (Sf21) [37, 38]. The marker genes were then encapsidated in the process of disassembly/assembly. Another study by Zhao *et al*. suggested that the packaging of the genome within papillomavirus pseudovirions also involves the interaction of the L2 protein with specific DNA sequences and these authors also demonstrated that the PsV capsid has the potential to encapsidate plasmids up to 10.2 kb in size [39].

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Rossi *et al*. utilized *Saccharomyces cerevisiae* to generate HPV16 pseudovirions [40]. In addition to the plasmid encoding the HPV16 capsid proteins, another target plasmid containing the green fluorescent protein gene was used to monitor the delivery of the plasmid into mammalian cells upon infection. The reporter gene expression could be analyzed by fluorescence activated cell sorting in vitro and detected by confocal microscopy *in vivo*. Thus HPV16 pseudovirions produced in yeast may be useful for both *in vitro* transduction and *in vivo* gene delivery. Buck *et al*. chose secreted alkaline phosphatase (SEAP) as the reporter molecule with signal

amplification in the presence of an appropriate enzymatic substrate [41]. The SEAP reporter plasmid which contains the SV40 origin of replication and codon-modified papillomavirus capsid genes, L1 and L2, were co-transfected into a 293 cell line, 293TT, that was engineered to express high levels of SV40 large tumor antigen (LT) to produce high-titer pseudovirions. The LT antigen unwinds DNA containing the SV40 origin of replication and initiates reporter plasmid replication. High titers (at least 10^6 pseudovirions from a 75 cm^2 flask of cells) can be routinely achieved due to the presence of SV40 origin of replication. The PsV encapsidating the SEAP reporter plasmid was used to develop a high-throughput *in vitro* neutralization assay with a 96 well plate format. Through this method, antibody-mediated PsV neutralization is detected by a reduction in SEAP activity. The SEAP activity was monitored using a highly sensitive chemiluminescent reporter system. This PsV-based neutralization assay was amenable for routine assays of large numbers of clinical samples with desired high throughput and high sensitivity due to enzyme turnover and chemiluninescence detection [26].

To further improve sensitivity, Bousarghin *et al*. developed a new method of HPV VLP-DNA complex formation to generate HPV16 and HPV31 pseudovirions [42]. This method was also sensitive for detecting the very low amount of neutralization antibodies after natural infection. It was also suitable for testing neutralization antibodies from human sera after vaccination.

Because the mechanism of a successful L1-based vaccine presumably relies on the *in vitro* generation of functional antibodies, the development and improvement of

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PsV-based neutralization assays has enabled the implementation of this assay in evaluation of neutralization antibodies after both immunization and natural infection.

Assessment of the neutralization activity of HPV antibodies

Prophylactic vaccines confer protection mainly through the elicitation of neutralizing antibodies. Therefore, the evaluation of neutralizing antibody levels is highly desirable for predicting vaccine efficacy. The development of *in vitro* HPV PsV-based neutralization assays has gradually met the need for the functional evaluation of human or animal serum samples post-vaccination. The PsV-based neutralization assay has been highly effective for quantifying potentially protective antibody responses against HPV acquired through natural infection and in prophylactic vaccine studies.

Yeager *et al*. demonstrated that HPV pseudovirions with β-lactamase as a reporter constitute a novel and efficient approach to detect and characterize HPV neutralizing antibodies. The results showed that no significant differences were observed between the HPV11 athymic mouse neutralization assay and the HPV11 PsV-based neutralization assay using a panel of sera samples with a range of titers differing by more than 300-fold. This also indicated that the PsV-based assay could detect the majority of functional antibodies elicited with the VLP immunization. The PsV-based neutralization assays were predominantly type specific for the tested serum. Significant cross-reactivity could only be observed between the closely types at the lowest serum dilution (1:100) such as HPV6 and HPV11 [27]. Pastrana *et al*.

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developed a sensitive high-throughput neutralization assay that was based on pseudovirions encapsidating a SEAP reporter gene [26]. This neutralization assay has been validated for HPV16 and HPV18 by analyzing the neutralization titers in serum samples of VLP-vaccinated women and women naturally infected with HPV. The results demonstrated that SEAP-based HPV neutralization assays directly measured the activity of antibodies relevant to vaccine efficacy and natural infection studies. Additionally, the HPV16 PsV-based neutralization assay is more sensitive than ELISA method when assessing antibodies in sera from unvaccinated women. The greater sensitivity was demonstrated by testing the neutralization activity of the sera from the individuals who were HPV16 DNA positive but HPV16 ELISA negative. Krajden *et al*. prepared the HPV16 and HPV18 PsVs using red fluorescent protein as a reporter [25]. They utilized this system to analyze the prevalence of HPV16 and HPV18 neutralization antibody levels in sera from prenatal pregnant women. Furthermore, HPV16 and HPV18 type-specific neutralizing antibodies from natural infection can be reliably measured and quantitated by the PsV-based neutralization assay. In addition, with the specificity of neutralizing antibody detection, the assay could be used in epidemiological investigation for the prior exposure population to vaccine types in order to optimize the use of the vaccine and monitor the antibody response induced by the vaccine.

The PsV-based neutralization assay is more reliable than the VLP-based ELISA as a measure of neutralizing antibodies. The PsV-based assay usually measures the functional neutralizing antibodies that are thought to be relevant to immune protection

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induced by vaccination.

To improve the type coverage and amenability for automation, Sehr *et al.* developed a high-throughput and automated neutralization assay with improved sensitivity for HPV16, 18, 31, 45, 52, 58 and BPV1 [43]. To fulfill a high throughput of the assay, the assay plate preparation was separated from the readout of the neutralization assay. Large batches of 384-well assay plates with serially diluted serum samples were prepared by an automated liquid handling system. This improved the throughput of the assay, making it more amenable for further automation. In addition, authors utilized the luciferase as the reporter. Compared to the SEAP, the use of the luciferase increased the sensitivity of the assay with higher signal to background ratios. The analytic sensitivity has been demonstrated for analyzing the World Health Organization international antibody standards in sera for HPV16 and HPV18. Accordingly, increased robustness and decreased assay variation was demonstrated, in addition to its improved sensitivity.

The correlation between the PsV-based neutralization assay and other serological assays used to evaluate the neutralizing antibodies was also investigated. Brown *et al.* assessed the correlations between the PsV-based neutralization assay and the competitive Luminex immunoassay and immunoglobulin G Luminex immunoassay [44]. In this assessment, subsets of serum samples were selected from three prior clinical trials of the quadrivalent HPV vaccine. The results showed that the three assays gave results that were highly concordant and reflected measurement of neutralization antibodies (or functional titer). Robbins *et al*. also compared the SEAP

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298 neutralization assay with both the competitive Luminex immunoassay (cLIA) and the
299 VLP-coating based ELISA [45]. The data demonstrated a good correlation among
300 these assays. Krajden *et al*. made another assessment of the HPV16 and HPV18
301 antibody response by the pseudovirion neutralization assay, Merck competitive
302 Luminex (Merck cLIA) and Merck total IgG Luminex (Merck TIgG) immunoassays
303 in a reduced dosage quadrivalent HPV vaccine trial [46]. The correlation between the
304 PsV-based neutralization assay and cLIA was better for HPV18 than for HPV16,
305 whereas the correlation between the PsV-based neutralization assay and total IgG was
306 similar for both of the viral types. This assessment indicated that the PsV-based
307 neutralization assay was more sensitive than cLIA and likely more specific than the
308 total IgG immunoassay.
309 Although good correlation among different assays has been demonstrated, the cLIA,
310 SEAP-NA, ELISA are biologically and technically different assays [45, 47]. They
311 measure the antibody response of serum in different aspects. The cLIA assay
312 developed by Merck was mainly used in the clinical trials to measure all the
313 neutralizing antibodies of all IgG classes in serum samples that compete for binding
314 to a specific epitope on VLP surface, e.g. V5 for HPV16, J4 for HPV18 with an
315 indication of a binding fluorescent signal strength. This assay is highly type specific.
316 Therefore, in a single assay the individual reactivity to multiple VLPs can be
317 simultaneously detected owing to the multiplexing capacity of the Luminex platform.
318 However, the major disadvantage is that it measures only the neutralizing antibodies
319 that bind the specific epitope, which make up a subset of the total antibodies elicited.

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Thus, this assay may underevaluate the potentially protective antibody response induced by the vaccine, which are non-overlapping with the tracing antibody.

SEAP-NA reflects the overall serum neutralizing antibodies by measuring a reporter gene product. This assay is considerably more laborious but recently it has been developed to be highly throughput and highly sensitive. Therefore, this assay is used primarily to correlate with the clinical protection potential of the serum samples.

The ELISA developed by GSK measures the antibodies that bind to a VLP antigen fixed on a solid face. Conformational heterogeneity of the surface immobilized antigen is an inevitable issue for plate based ELISAs. The signal was detected by adding a secondary antibody conjugated with an enzyme e.g. alkaline phosphatase. It has the disadvantage of potentially detecting the nonneutralizing antibodies elicited by the VLPs.

The PsV-based neutralization assay is still the gold standard among various assays for evaluating the potentially protective antibodies. The implementation of the PsV-based neutralization assay is of the utmost importance in the evaluation of clinical serum samples to demonstrate the efficacy of prophylactic HPV vaccines. Comparison and correlation could be better determined by the first antibody standard established by WHO Expert Committee on Biological Standardization [48].

Different binding sites for neutralizing antibodies to the HPV capsid

Natural infection with HPV or vaccination with HPV L1 VLP-based vaccines can induce a complex antibody response. A wide array of different antibodies directed to

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different epitopes presented on the VLP surface can be elicited by the virions or VLP-based immunogens. The neutralizing antibodies usually exert their function by blocking the binding and entry of HPVs into host cells. HPV16 binding to heparin sulfate proteoglycans (HSPGs) on the basement membrane was firstly reported by Johnson *et al*. using a murine cervicovaginal challenge model *in vivo*. Additionally, initial HPV16 binding occurs via heparin sulfate proteoglycans (HSPGs) located either on the epithelial cell surface or on the basement membrane was also verified *in vitro* by Knappe *et al*. and Richards *et al*.[49-51]. HSPGs were first described as HPV binding receptors using HPV11 L1 VLP on HaCaT cells [52, 53]. The other studies showed that HSPGs were essential for infection using HPV16 PsV and HPV33 PsV on COS-7 cells [49, 54]. The exterior surface of HPVs is mainly composed of the L1 capsid protein with 5 surface loops. HPV16 L1 residues Lys278 and Lys361 on the top of the pentamer were identified as the primary attachment sites to HSPGs, which demonstrated a direct interaction between HPV16 L1 and HSPGs [50]. Thus, the interaction between neutralizing antibodies and the major capsid protein can block the virions from binding to the target host cells, thus inhibiting the subsequent cell entry process.

Various neutralizing mAbs have been isolated by different labs to further elucidate the neutralization mechanism and the specific binding sites of neutralizing mAbs. Three different binding modes for neutralization antibodies on the viral capsid have been determined to date (Figure 2). Bovine papillomavirus and human papillomavirus are in the same genus and are highly similar to each other, particularly in regards to

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their capsid surface structure [55]. Therefore, BPV has commonly been used as a surrogate to study the biological profile of HPV. Two immunocomplexes of the BPV1 major capsid protein L1 and two neutralizing mAbs were analyzed by electron cryomicroscopy (cryoEM) and three-dimensional image reconstruction to 13 Å resolution [56]. mAb #9 is representative of a set of neutralization antibodies that can inhibit viral binding to the host cell surface. In the cryoEM structure, mAb #9 bound to the major L1 molecules of both pentavalent and hexavalent capsomeres. The epitope of mAb #9 was located in the outer tips of capsomeres (Figure 2). The other neutralizing antibody, mAb 5B6, can efficiently neutralize the papillomaviruses without significantly inhibiting viral binding to the host cell surface. Thus, this type of neutralizing antibody may neutralize the virus by preventing capsid uncoating and thus blocking the release of viral DNA into the nucleus. Structural analysis showed that mAb 5B6 did not bind to pentavalent capsomeres, which reflected the significant structural and environmental difference of its epitope between the pentavalent and hexavalent capsomeres [56]. A similar observation was made for the different conformations of pentavalent versus hexavalent capsomeres. The epitope localization showed that mAb 5B6 bound both monovalently and bivalently to the sides of hexavalent capsomeres approximately two-thirds of the way down from the outer tips (Figure 2). Other work on recombinant vaccine VLPs by Zhao *et al*. demonstrated two different binding models for two different neutralizing antibodies (H11.B2 for HPV11 and H16.V5 for HPV16) using cryoEM image reconstruction [57, 58]. Both H16.V5 and H11.B2 showed highly efficient neutralization activity in the

pseudovirion neutralization assay. These antibodies also recognized immuno dominant epitopes when analyzed by competition assay using human sera from naturally infected individuals. The cryoEM structure of the VLP-Fab complex in comparison to VLP alone revealed that the binding site of mAb H11.B2 was located at the center of the capsomere, coincident with the L2 binding sites in the capsid, indicating 72 potential binding sites per capsid [57]. However, the other well-studied mAb H16.V5 with an extremely high neutralization efficiency showed a different binding mode on the apex of the capsomere [57, 59]. H16.V5 bound only to capsomeres with six neighboring capsomeres (at the 3-fold axes of symmetry), but there was no evidence from the density map for its binding to the pentavalent capsomeres (at 5-fold axes of the symmetry) (Figure 3). Based on the cryoEM structures, there are 300 potential binding sites for mAb H16.V5 due to steric hindrance or different structures between hexavalent versus pentavalent capsomeres in the recombinant icosahedral VLPs.

A recent study by Lee *et al*. also determined the spatial structure of the immunocomplex of H16.V5 and HPV16 pseudovirions [59]. The complete epitope of H16.V5 was identified using cryoEM and 3D image reconstruction. Based on the experimental methods using the model of VLP-Fab complexes, the H16.V5 epitope was found to be composed of the antigenic loops BC, DE and EF, in addition to the previously identified epitopes located in the FG loop and HI loop. The study indicated that the binding of H16.V5 Fab induced a global conformational change. The study showed that the H16.V5 Fab bound to the hexavalent capsomeres as well as to the

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pentavalent capsomeres. H16.V5 Fab also bound preferentially to the hexavalent capsomeres over the pentavalent capsomeres (Figure 3). This preferred binding suggested that the affinity for the two binding sites was different and that this phenomenon might exist intrinsically or be induced by H16.V5 Fab binding. In addition, structural consolidation in the base of the capsomere as well as on the surface flexible loop was observed by the binding of H16.V5 to the capsid (Figure 3).

The other recent study by Guan *et al*. showed that Fab fragment from three additional HPV16-specific antibodies, mAbs H16.1A H16.14J and H263.A2, had complex and overlapping epitopes similar to the H16.V5 epitope [61]. CryoEM image reconstructions showed that the DE loop formed the core of each mAb footprint, and that the mAbs also bound to the FG, HI, DE and EF loops, although the participation of the latter loops varied between the antibodies (Figure 3).

In addition to the roles played by major capsid protein L1, the minor capsid protein L2 was also determined to be involved in the viral cell entry process, a critical step for viral infection. A subtle conformational change is triggered by the interaction between HSPGs and the HPV capsid that further leads to the exposure of an amino-terminal portion of L2. Subsequently, the cleavage of L2 by furin is required for further infection, which results in additional changes to the capsid conformation. More importantly, during this furin cleavage process, a broadly neutralizing epitope of the L2 protein is exposed. The exposure of this epitope was first characterized with the neutralizing mAb RG-1 [22, 62].

The observation of different binding modes for various neutralization antibodies

against the major capsid protein L1 or the minor capsid protein L2 at a higher resolution would shed light on the mechanisms of how HPV virions could be neutralized during cell attachment or entry. This will likely soon be achievable with the improvement of direct electron detectors and image reconstruction software in cryoEM.

The encouraging clinical trial results and long-term follow-up results have validated the efficacy of the launched vaccines (Cervarix®, Gardasil® and Gardasil®9). The protective immunity conferred by vaccination is due to the presence of functional epitopes in the vaccine antigens. Neutralizing antibody responses are believed to be the most direct evidence of vaccine efficacy and the primary mechanism of vaccine-induced protective immunity [63]. Therefore, integrity of the correct spatial conformation and sufficient exposure of the functional epitopes are essential during the bioprocessing of vaccine antigens. Some epitope-based assays were designed and developed to evaluate antibody response in clinical sera to verify vaccine immunogenicity. Secreted alkaline phosphatase neutralization assay has been used to quantify the neutralizing antibody response elicited by Cervarix® and Gardasil® [12, 26, 64-67].More recently, serological assays for an *E.coli*-derived VLP-based vaccine were performed with a slightly different format of PsV-based assay [68-70]. For Gardasil®, the competitive Luminex immunoassay was developed to measure neutralizing antibodies with high-sensitivity by competing for binding to one epitope, e.g., H16.V5 for HPV16 and H18.R5 for HPV18. [71-74]. With a better understanding of the functional epitopes, more epitope-based assays will be

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reasonably designed [75]. Therefore, the accurate epitope determination at molecular level will shed light on assay developments to monitor the manufacturing process of vaccines and to evaluate the effective antibody response in clinical sera.

Discussion

Currently, there are two prophylactic HPV vaccines on the market, the bivalent vaccine containing the VLP antigen for HPV16/18 (Cervarix® from GSK) and the quadrivalent (or recently-licensed nonavalent) vaccine against HPV6, 11, 16, and 18 (with 31, 33, 45, 52, 58) from Merck. Both vaccines are based on VLPs as the active component, produced by expressing the L1 protein using recombinant technology. The expressed L1 proteins self-assemble into VLPs that resemble the native virions with specific spatial structural features. This high degree of resemblance of the VLPs to viral capsids is the structural basis for their ability to elicit neutralization and functional antibodies for prevention of viral infection. Two issues are the questions being discussed when licensing a new biosimilar HPV vaccine: *a) what is the minimal level of neutralizing antibody required for protection? b) what endpoint(s) should be used for evaluation of a new HPV vaccine – clinical (i.e., CIN2+), persistent infection or virological endpoint?* The former question is difficult to address due to the high efficacy of the vaccines. For the latter questions, consensus has been reached gradually among scientists that persistent infection is the pre-requisite for CIN1, CIN2 and higher precancerous lesions, thus, using endpoints other than the initially used "CNI2+" for Gardasil and Cervarix is feasible. Based on what has been learned since the initial licensure of Gardasil in 2006, a trove of data has been accumulated in the

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understanding of the vaccine efficacy and endpoints that can be used for the evaluation of HPV vaccines: persistent infection (>6 Mo) or virological endpoints (such as virus neutralization activity of serum samples using the PsV-based neutralization assay). This has been reflected in the WHO guidelines for licensing existing or new HPV vaccines in their countries/regions by the respective national regulatory agencies [Recommendations to assure the quality, safety and efficacy of recombinant human papillomavirus virus-like particle vaccines. *WHO Expert Committee On Biological Standardization* 2015: http://www.who.int/biologicals/BS2252_HPV_Recommendations_30062015_tz.pdf?ua=30062011].

The presence of functional neutralizing antibodies is the basis for conferring protection against HPV infection. Neutralizing antibodies played a key role in preventing HPV infection by blocking attachment to cells or inhibiting the cell entry process depending on the specific epitopes on the viral capsid they recognize. The major capsid protein, L1, harbors most of the neutralizing epitopes. This is also the basis for the function of the currently licensed L1-based vaccines. However, some reports showed some neutralizing Abs could be elicited by recombinant L2 protein. Since L2 has higher degree of amino acid sequence homology as compared to L1, it is also a target for broad spectrum vaccine development. Since most PsV systems contain both L1 and L2, therefore the same PsV-based neutralization assay can be used to assess the function of the Abs elicited by either L1- or L2-based vaccines.

In addition, the PsV neutralization assay could provide an alternative method in

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bioprocessing for VLP quality analysis for assuring the presence of neutralization epitopes on antigens. This analysis can be carried out with *in vivo* mouse potency assays. Mouse potency was used to evaluate the immunogenicity of vaccines with the indication of the ED_{50} value. Specifically, mice were immunized with serially diluted vaccine and the serum samples were collected four weeks after immunization. Subsequently, the anti-vaccine antigen antibodies were detected in an antigen-coating ELISA assay. The ED_{50} value was calculated with indication of the {something missing here} that would result in 50% seroconversion for the tested animals [82]. The titer of neutralization antibodies reflected of the level of functional Abs elicited by immunization with immunogens harboring conformational and functional epitopes. For many infectious diseases, assays to detect neutralizing antibodies are used to assess the immunogenicity of prophylactic vaccines. *In vitro* PsV-based neutralization assays involve measuring the inhibition of HPV pseudovirion binding and infection of cultured cells by employing a reporter gene to score the level of infection.

Due to the type-specificity of the current L1 VLP-based vaccines, a broad-spectrum next generation HPV vaccine needs to be developed that would prevent the infection of more additional HPV types for broader type coverage. A nonavalent vaccine, with L1 antigens of HPV6, 11, 16, 18, 31, 33, 45, 52, and 58, was recently licensed in the USA (Gardasil®9 developed by Merck) [83-85]. The HPV capsid contains the major L1 protein and the minor L2 protein. The L1 protein is abundantly present on the viral surface, which harbors the immunodominant epitopes inducing strong B cell

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responses. However, the L2 protein is barely visible to the immune system. Only when the virus binds to the basement membrane of host cell does L2 become exposed due to conformational changes in the capsid[50, 86]. Its neutralization epitopes are deemed to be transiently exposed after furin cleavage in the viral infection process. The process was induced by the interaction between L1 and HSPGs that triggers a conformational change of the capsid. It is the transient exposure that induces the immune response to generate the specific antibodies. Several cross-neutralizing antibodies have been identified and studied extensively, such as RG1 recognizing residues 17-36 of L2 and the other two antibodies K4L2 and K18L2 targeting the sites located at residues 20-38 of L2, which are exposed after furin cleavage [22-24].Therefore, the L2 protein is also a candidate for a broad-spectrum HPV prophylactic vaccine with the conserved epitopes located at the N-terminus of the L2 protein. Recently, efforts have been made to develop an L2-based prophylactic vaccine for the development of the next generation of HPV vaccine, which has had some success [87-91].

Understanding of the clinically relevant epitopes is critical for vaccine design and quality control in the manufacturing process of vaccines. The mAbs recognizing immuno dominant epitopes are useful molecular tools. The binding activity to the neutralizing mAbs can serve as a surrogate marker for VLP antigens. Therefore, mAbs can be utilized for process monitoring and product assessment to ensure the product consistency for VLP-based vaccines. More specifically, the entire conformational epitope recognized by the mAb HPV16.V5 was recently identified by

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663 Lee *et al*., and this work also advanced the understanding of the neutralization
664 mechanism of the well-studied HPV16.V5 [59]. This mAb was also used in the
665 clinical serology assay for Gardasil® in the competitive Luminex-based immunoassay,
666 where the effectiveness of competition of the serum samples with H16.V5 was used
667 as a surrogate marker for vaccine efficacy against HPV16 [45, 60, 71-73]. The
668 binding of HPV16.V5 to the capsid resulted in a significant conformational change
669 and structural consolidation that altered the capsid-Ab complex. This binding ordered
670 the apical loops of HPV16 and consolidated the "invading-arm" structure. In addition,
671 this conformational change was transmitted to the lower region of the capsomere and
672 tightened the intercapsomeric connection at the capsid floor.

673 A similar phenomenon of conformational changes induced by mAb binding was
674 also observed in other viral capsid-Ab complexes. The neutralizing mAb 1A1D-2
675 strongly neutralized dengue virus types 1, 2, and 3. The binding of this mAb to the
676 envelope glycoprotein E of dengue virus altered the arrangement of the surface
677 glycoproteins. The Fab molecules might capture the transient viral intermediate
678 conformation, further hiding the 1A1D-2 epitopes exposed for Fab binding. This
679 change is likely the potential mechanism by which this neutralization antibody could
680 inhibit viral attachment to host cells [1]. Another example is the binding of
681 neutralization mAb E18 to the mature human enterovirus 71 (EV71), which induces
682 the conformational change in the capsid. mAb E18 recognized the conformation of
683 empty and immature particles of EV71, which are similar to the "A" particles when
684 the EV71 recognizes a potential host cell before genome release. The binding of E18

to mature virions induced a conformational change that transformed the infectious virions into “A” particles. This mechanism demonstrated that mAb E18 could neutralize the virus by inducing genome release. Therefore, such a characteristic made the E18 a potential therapeutic Ab candidate [2].

In summary, the PsV-based neutralization system has been broadly used in the efficacy evaluation of neutralization antibodies generated by vaccination or natural infection. Antibodies could exert their viral neutralizing functions via different mechanisms. These include blocking the binding between viruses and cell receptors and altering the epitopes recognized by the cell receptors by inducing conformational changes in the viral capsid. Three binding modes for HPV capsid and mAb were determined using cryoEM image reconstruction techniques at intermediate resolution. Higher resolution structural determination will aid in the understanding of the epitope details and the functional mechanism of neutralization mAbs. In addition, better knowledge regarding the mAb neutralization mechanism will advance process understanding and process control during bioprocessing in vaccine production and in the design and development of the next generation vaccines [30].

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Figure Legends

Fig. 1 The different forms, natural virion, pseudovirion, and VLP of papillomavirus and their applications. (A) Surface representation of the BPV cryoEM image reconstruction. The native virions were used in biological studies on viral assembly and cell entry, among others. (Adapted from [56]) (B) CryoEM image reconstructions of HPV16 pseudovirions that were used to develop the PsV-based neutralization assay. (Adapted from [92]) (C) CryoEM image reconstructions of HPV16 VLPs. Two VLP-based prophylactic vaccines have been globally approved for clinical use. (Adapted from [57]) Native virion and pseudovirion are composed of the major L1 and the minor L2 proteins. They also both encapsidate DNA. However, VLPs do not contain the L2 protein, which is self-assembled from L1 protein only without DNA involvement. The structures of all three forms have been determined and they all showed icosahedral symmetry. Although the native virion and pseudovirion contain the L2 protein, the precise structure of L2 is poorly resolved in all the available structures.

Fig. 2 Three binding modes for neutralizing antibodies against papillomavirus capsid. (Left) mAb H11.B2 binding to HPV11 VLP (the DE loops on L1) indicates that the binding sites are located at the center of the capsomere. (Middle) mAb 5B6 binding to the two L1 molecules of the adjacent hexavalent capsomeres. (Right) mAb #9 binding to the outer surface of the hexavalent capsomere. mAb H16.V5 binding to HPV16 VLP (FG and HI loops on L1) shows that H16.V5 only preferentially binds to the

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outer surface of the hexavalent capsomeres. The inset pictures were used to further clarify the interaction models between antibody and antigen.

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Fig. 3 HPV16.V5 binding sites and specific amino acid residues involved at the Ab-Ag interface. (A) The footprint of antibodies H16.V5, H16.1A H16.14J and H263.A2 mapped to the stereographic projection of a capsomer. The virus surface was represented as a quilt format for the icosahedral symmetric unit, with the polar angles φ and θ representing the latitude and longitude. The color bar indicates the different antigenic L1 loops and each mAb epitope was denoted using dashed circle. The common epitope indicated by arrow includes all residues identified in each mAb footprint. (Adapted from [61]) (B) A 3D reconstruction structure based on cryo-EM data of mAb H16.V5 binding to HPV16 VLP (FG and HI loops on L1) shows that H16.V5 only binds to the hexavalent capsomeres but not the pentavalent capsomeres. (EMDB:28370). (Adapted from [57]) (C) The 3D structural model of mAb H16.V5 binding to HPV16 VLP, with magenta for H16.V5 binding site on FG loop and red on HI loop, and cyan for VLP. (Adapted from [30]) (D) The HPV16.V5 Fab binds 17 residues across five loops from two neighboring L1 proteins. The contacting residues are shown as spheres with the residue names and loops labeled. Within the five loops, three loops, BC, DE, FG, are contributed by the first L1 protein. The other two loops, DE and HI are contributed by the second neighboring L1 protein. (Adapted from [59]) (E) The accurate epitope location of HPV16.V5 on the exterior flexible loops with a total of identified 16 amino acid residues. The loops with red represent the major

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contribution from one L1 molecule. The blue HI loop confers the minor interaction by a second neighboring L1.

BPV, Booy et al, JMB, 1998

BPV, Wolf et al, PNAS, 2010

CRPV, Belnap et al, JMB, 1996

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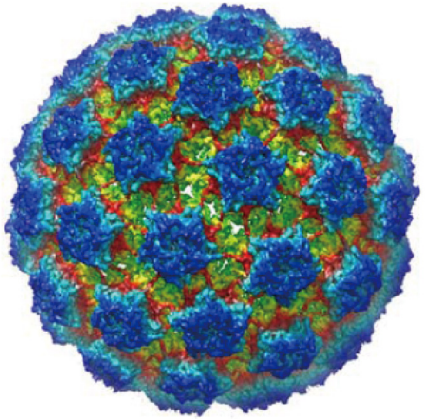
A



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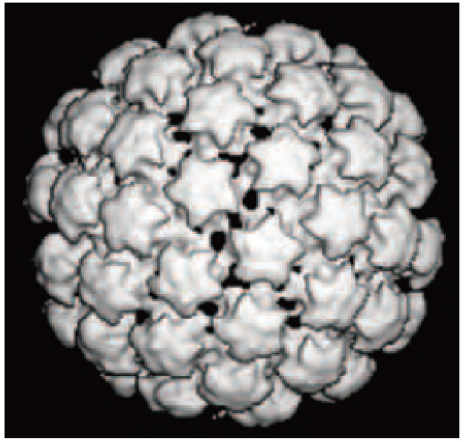

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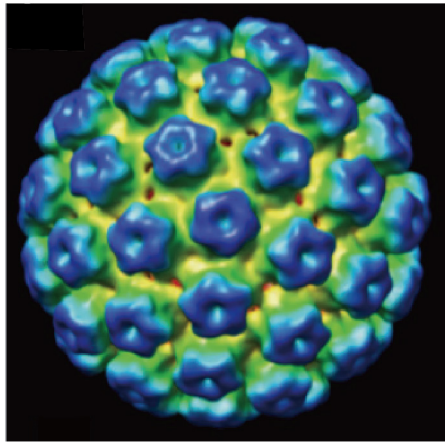
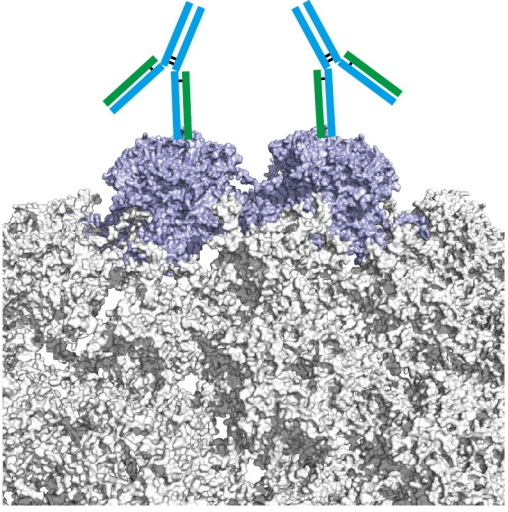
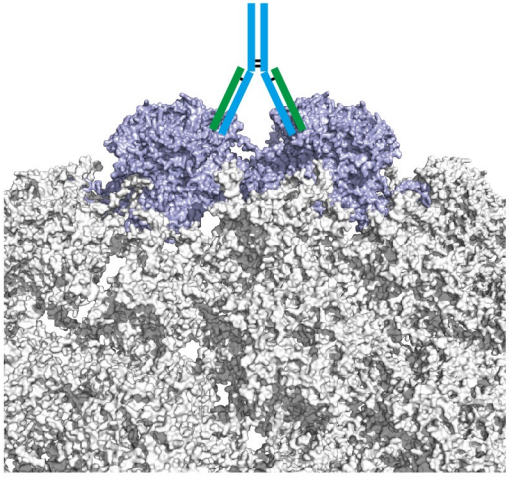


DNA	+	+	—
Application	Biological studies including viral assembly, cell entry and others	Neutralization assay	Prophylactic vaccines

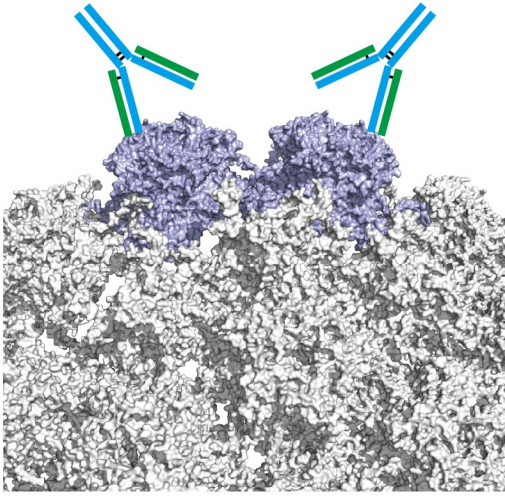
Fig. 1



H11.B2, Zhao *et al*, HVI, 2014



mAb 5B6 Booy *et al*, JMB, 1998

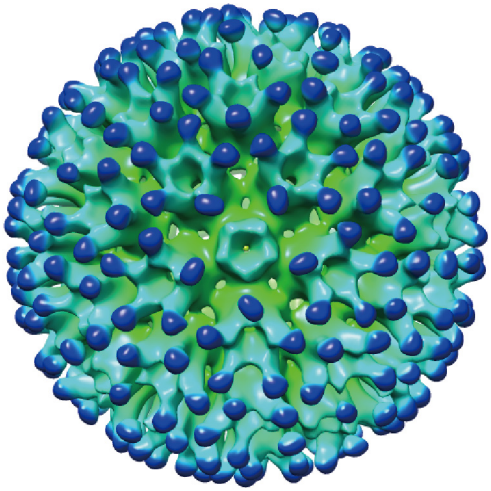


mAb #9, Booy *et al*, JMB, 1998

H16.V5, Zhao *et al*, HVI, 2014

Fig. 2

A



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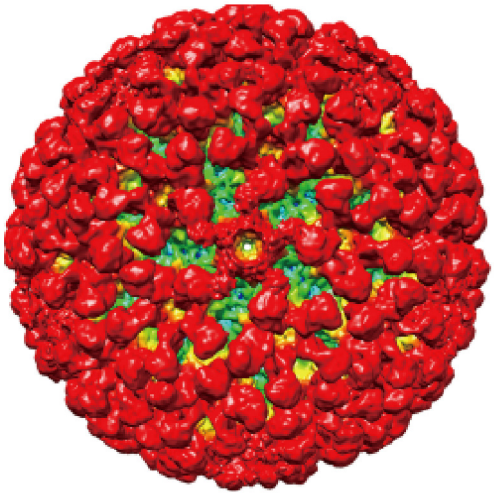

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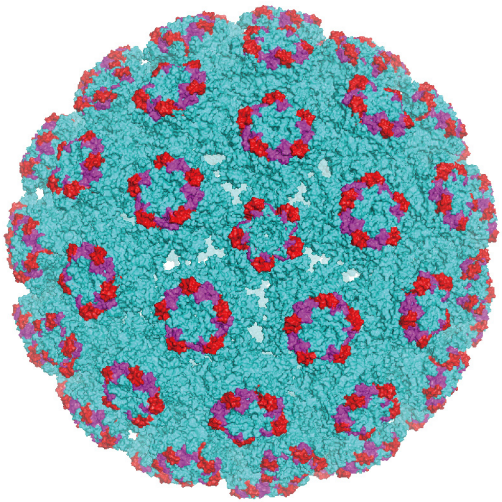

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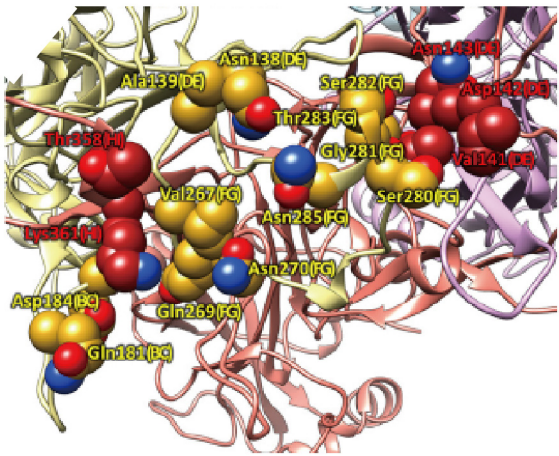
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Ser282(FG)
Asp142(DE)
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Thr358(HJ)
Gly281(FG)
Val141(DE)
Val267(FG)
Ser280(FG)
Asn285(FG)
Lys361(HJ)
Asn270(FG)
Asp184(BC)
Gln269(FG)
Gln181(BC)

Fig. 3