

**IMMUNOLOGY AND VACCINOLOGY OF  
HUMAN PAPILLOMAVIRUS  
MINOR CAPSID PROTEIN L2**

by  
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**Abstract (341/350 words)**

Human papillomavirus (HPV) minor capsid protein L2 has potential in being a broad spectrum protective yet low cost vaccine that is needed to eliminate HPV-related cancer as a global health problem. Several L2-based vaccines designed to induce broadly neutralizing serum antibodies are nearing phase 1 clinical trials to assess their safety and immunogenicity. Therefore, robust tools for serological assessment of L2-specific antibody responses to infection and vaccination are required. Presently, HPV neutralizing antibodies are measured *in vitro* using HPV Pseudovirions that deliver a reporter plasmid rather than an infectious genome. However the current assays are either cumbersome or lack sensitivity for L2-specific neutralizing antibodies because they do not fully replicate *in vitro* the slow but critical cleavage of L2 by furin observed *in vivo*. To address this issue, we developed a high-throughput *in vitro* neutralization assay based on a furin-cleaved infectious intermediate and validated it as sensitive measure for both HPV L1 and L2-specific neutralizing antibodies of both human and animal origin. We also generated two human chimeric antibodies for use as a standard and/or positive control necessary for validation of immunogenicity studies of planned clinical trials of L2-based vaccines. Further mechanistic studies of L2-antibody mediated protection against experimental viral challenge demonstrated the importance of the Fc region of the antibody in mediating phagocyte recognition of virions. Finally, because vaccination against L2 alone does not provide therapeutic benefit against established infection, L2 was fused with early viral antigens as an approach to combine therapy and protective immunity. Orthotopic tumor lines, such as the TC-1 cell line generated by ectopic expression of HPV16 E6 and E7 and mutant ras in C57BL6 mouse lung fibroblasts, have been utilized to assay therapeutic immunity. However, these lines do not emulate the viral life cycle or papilloma development. Given the tractability of mouse models and a plethora of reagents, we have exploited a recently identified laboratory mouse papillomavirus model (MusPV1) and pseudovirion challenge studies to demonstrate the potential of E6E7L2 fusion vaccines to prevent new infections and for the treatment of established papillomavirus infection or disease.

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***“You can just hang around outside in the sun all day, tossing a ball around, or you can sit at your computer and do something that matters! “***

*Eric Cartman "South Park: Make Love, Not Warcraft (#10.8)" (2006)*

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## **A. Introduction**

### **a) Scope of thesis**

Human Papillomaviruses (HPVs) are a major global health issue. Although the majority of the over 120 genotypes which have been fully characterized [1] cause benign disease, persistent infection by any one of a subset of 15 “high risk” or oncogenic Human papillomavirus (hrHPV) is a necessary although insufficient causal factor for cervical cancer as well as a subset of anogenital and oropharynx cancers[2, 3]. The remaining types are considered “low risk” and are divided in to those targeting the skin (cutaneous types) or the mucosa. Even the benign types can be associated with considerable morbidity, including reoccurring warts on genital and non-genital skin areas that are costly and often painful to treat, although HPV infections can be asymptomatic. HPV-associated disease is often more pronounced and recalcitrant in immune-compromised patients such as HIV+ patients or solid organ transplant recipients [4] suggesting the importance of immune control and the promise of immunotherapies.

Following the success of bivalent (Cervarix) and quadrivalent (Gardasil) prophylactic HPV vaccines based on L1 virus-like particles (VLP), a nine-valent (Gardasil-9) version has just been licensed based on >90% efficacy against 7/15 of the most prevalent oncogenic types and 2 types that cause 90% of all genital warts cases has been recently approved by the FDA[5]. However, even this 9-valent vaccine does not provide truly comprehensive protection and its high manufacturing costs are likely to limit widespread use in developing countries with limited resources, no screening infrastructure and the highest rates of cervical cancer.

The HPV minor capsid protein L2 is a promising vaccine antigen as pre-clinical vaccination studies highlight its ability to elicit serum antibody that cross-neutralizes diverse HPV types[6]. Further, the cross-neutralizing epitopes are linear and thus can be produced in bacteria, a less expensive system compared to the licensed HPV vaccines that require eukaryotic expression systems to produce L1 VLP. Several L2-based candidate HPV

vaccines have since been developed and are being produced under cGMP for early phase clinical trials [7-10]. However, the lack of suitable positive controls for validation and high throughput functional assays for measurement of L2-specific neutralizing antibodies at high sensitivity have hampered the development of these L2-based candidate HPV vaccines [11].

Importantly, neither the licensed medical vaccines nor L2-based vaccines have been demonstrated to provide therapeutic value. Since there are currently no licensed anti-virals against papillomaviruses, there remains a major need to develop immunotherapies to treat persistent HPV infections and disease.

Here, we begin with an introduction to the biology of papillomavirus L2 (based on our literature review [6]) followed by the impact of HPV vaccines since licensure in 2006 (also derived from our review [12]). The current state of development of second generation prophylactic and therapeutic vaccines will then be introduced [12] to provide context for the experimental studies within this thesis. The experimental studies conducted are described in five chapters. In Chapters 1-3, we describe the isolation and characterization of an infectious intermediate (Chapter 1), its use to develop a high throughput and sensitive *in vitro* neutralization assay (Chapter 2) and validation of the assay methodology, including the development of human chimeric monoclonal antibodies for this purpose (Chapter 3). These new methodologies were developed to support proposed phase 1 clinical trials of L2 vaccines [13, 14]. An investigation of how the Fc domain of L2-antibody contributes to protection from experimental challenge will then be presented (Chapter 4), followed by immunologic studies of an E6E7L2-based vaccine in the MusPV1 disease model (Chapter 5).

## **b) L2, the minor capsid protein of papillomavirus**

### **Introduction**

Human papillomaviruses (HPV) are the etiologic agent of 5% of all lethal cases of cancer worldwide, and even benign infections can cause considerable morbidity for patients and expense to the healthcare system. While the oncogenic HPV genotypes are the best studied, many fundamental observations have first been made with animal papillomaviruses [2].

Productive infection by papillomaviruses occurs in many vertebrates and exhibits a strict host tropism and a requirement for epithelial differentiation. Animal papillomaviruses, notably cottontail rabbit papillomavirus (CRPV), bovine papillomavirus (BPV), rabbit oral papillomavirus (ROPV) have been invaluable models for virologic, and especially vaccine studies. These animal papillomavirus models remain important because of many technical challenges associated with the oncogenic HPV genotypes; HPV cannot be grown in cultured cancer cell lines, obtaining virions of oncogenic HPV genotypes from clinical lesions is not feasible, infection is not lytic (and thus fails to produce a readily measurable phenotype), and finally infection of animals with HPV does not produce lesions due to strict host tropism.

Over the years, many of these hurdles have been circumvented through the use of organotypic raft culture to produce native virions [15, 16], or the codon optimization of the capsid genes enabling robust L1 and L2 expression for the formation of pseudovirions (PsV) carrying a reporter construct [17] that can be used to challenge mice or even primates [18, 19]. While much of the early seminal work was performed with animal papillomavirus, in particular Bovine papillomaviruses (BPV1), fortunately there appears to be a strong conservation of both the domains and functions of L2 across the *Papillomaviridae* which allows consolidation of findings on PV L2.

At present, there are over a hundred known HPV genotypes that are categorized into 5 genera (alpha, beta, gamma, mu and nu) based upon their genomic sequence [1, 20]. They can also be subdivided based on their tropism for either cutaneous or mucosal epithelium respectively,

as well as their association with cancer or solely benign lesions. Cutaneous HPV types are typically “low-risk” as their infections are either asymptomatic or result in self-limited and benign tumors, as seen for the gamma types such as HPV1 and HPV2 that cause warts. The beta types, notably HPV5 and HPV8, are typically asymptomatic in healthy individuals, but have been associated with non-melanoma squamous cell carcinoma (SCC) in patients with epidermodysplasia verruciformis (a hereditary condition resulting from germline mutations in EVER1 or EVER2), or upon immune suppression due to HIV co-infection or drug treatment upon solid organ transplantation. Most mucosal HPV types are also benign, such as HPV6 or HPV11, but a dozen or so “high risk” or “oncogenic” genotypes are carcinogenic. While the majority of oncogenic HPV infections are self-limited and subject to immune clearance, persistent infections are associated with a dramatically increased risk for the development of cervical, other anogenital and/or oropharyngeal cancers. HPV types 16 and 18 account for 50 and 20% of all cervical cancer cases respectively. Other high/intermediate risk types such as HPV 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 constitute the remaining 30% HPV-associated malignancies and are associated with a slower onset of dysplasia [21]. Importantly, HPV16 is even more predominant (~90%) in other anogenital and oropharyngeal malignancies [2, 22]. Given its disproportionate impact on human health, most studies of L2 biology at present are concentrated on HPV16.

The circular and double-stranded papillomavirus genome encodes six ‘early’ proteins (E1, E2, E4, E5, E6, and E7) and two ‘late’ proteins (L1 and L2), subdivided based on their spatial-temporal expression pattern during the virus life cycle. The ‘early’ proteins can be further sub-divided into two regulatory genes involved in replication and transcription (E1 and E2), 3 oncogenes (E5, E6 and E7), and E4 which contributes to virion production and actually exhibits an expression pattern closer to the late proteins. The late proteins L1 and L2 are structural components of the viral capsid [23]. Expression of L1 and L2 is not detected in infected basal epithelial cells, but they are both detected in the nuclei of the terminally differentiated cells in the uppermost layers of the squamous epithelium, appearing first even

later (i.e. higher in the epithelium) than E4. When produced in a variety of recombinant expression systems, L1 can self-assemble, to form empty virus-like particles (VLPs) that are the basis of the licensed HPV vaccines [24]. L2 does not form VLPs but can be incorporated when co-expressed with L1. In this review, we will be focusing on the biology of the minor capsid protein, L2, which plays a key role both during virion assembly and the infectious process.

### **The L2 protein**

L2 is just under 500 amino acids in length, which corresponds to an estimated molecular mass of approximately 55KDa. However, L2 typically exhibits an apparent molecular weight of 64-78kDa by SDS-PAGE analysis [25-28]. The reason for this phenomenon is unclear as there are no known post-translational modifications of L2 (with the exception of modification by SUMO of lysine 35 of HPV16 L2 [3]), and a similar size is observed for L2 produced in bacteria. Rose *et al* reported that L2 in native HPV11 virions exhibited a doublet, but the size of L2 was not impacted by glycosylase treatment, suggesting that glycosylation was not a factor, and the lower molecular weight form might instead represent proteolytic cleavage [29]. L2 has several key functional roles and numerous interacting partners (Table 2). The main domain sequences used by L2 have been mapped through deletion and/or mutagenesis studies using HPV16 L2 (Figure 1). Hence, much of our discussion and analysis here will be based mainly on HPV16 L2's primary sequence and these mapped domains because very little information exists on its higher order structures.

### **Structural Biology: L2 in the context of the papillomavirus capsid**

The basic architecture of the papillomavirus virion is a non-enveloped T=7d icosahedral capsid with a diameter of 55-60nm (Figure 2A). Each virion contains 360 L1 proteins. L1 first stably assembles into star-shaped units of five called capsomers (sometimes termed 'pentamers') that have a central donut-like cavity, and the capsid is formed by the association of 72 such capsomeres via interlocking arms and di-sulfide bridging. The virion's capsid also



contains an ill-defined number of L2 proteins; although up to 72 L2 proteins have been estimated in a single capsid (1:5 L2 to L1 proteins ratio)[22, 30, 31]. Smaller amounts have also been described in preparations of native virions purified from bovine and human warts, e.g. 8% [26], 3% [32] or 2-5% [33]. These lower percentages may reflect a naturally lower occupancy, proteolytic degradation and/or the presence of empty particles in the preparations from warts, as the latter have been associated with lower fractions of L2.

Studies by Finnen et al on HPV11 revealed that there is a L1 binding site on the carboxy-terminus of L2 (region 396-439 on HPV11 L2). This region is characterized by several proline residues (PxxP) and it has been shown that such PxxP motifs have been associated with protein-protein interactions in other systems. Similar PxxP motifs were observed in a similar region of the C-termini of other L2 thus suggesting that this is a well conserved L1-binding site on PV L2 (Figure 2D). As binding of L2 to L1 was not affected significantly under conditions of high salt, weak detergents, urea and pH, it appears that the main mode of L1:L2 interaction is hydrophobic in nature. However, the L1 residues mediating the interaction with L2 in the capsid remain undefined, although some speculative modeling has been done based on the available X-ray crystallographic structure of L1 VLPs which suggest that L2 might interact with L1 via the N-terminus [30, 34]. Also, purified L1 VLP and L2 are unable to form complexes *in vitro* without co-expression and this suggests that their interaction must occur prior to capsid assembly or at the capsomer level rather than insertion of L2 into a formed L1 VLP [31, 35].

The amino terminus of L2 contains two highly conserved cysteine residues (C22 and C28) (see Figure 5) across all PV types which form an intra-molecular disulfide hairpin loop rather than bridging with L1. Studies using HPV16 PsV showed that the point mutation of either or both cysteine residues resulted non-infectious virions but did not affect virus capsid assembly. However, this effect was not observed for BPV1 PsV [36, 37]. Interestingly, using PV made from organotypic raft cultures, Conway *et al* found that mutating these cysteine residues

improved infectivity compared to wild type. The authors suggested that this remarkable difference could possibly be attributed to differences in virion preparation producing subtle differences in capsid structure between PV made in cell culture versus differentiating epithelial tissue. In addition, it was suggested that these cysteine residues potentially play a key role in late stage capsid stabilization by modulating the accessibility of L2 on the surface of raft-derived virions [38].

At present, studies in the literature using antibodies suggest that PV L2 is predominantly hidden below the surface of native virions although its configuration remains unknown. A detailed study using antisera to overlapping peptides covering the entire HPV16 L2 sequence suggested that several regions 32-81, 212-231, 272-291 and 347-381 are accessible on the surface of HPV16 L1/L2 VLPs [39]. However, in another study using several monoclonal antibodies raised against BPV1 L2 protein, it was suggested that only region 61-123 was actually exposed on the surface [40]. Likewise certain studies mapping neutralizing epitopes to HPV 16 (see below in immunology section) suggest that only the first amino terminal ~120 amino acids are available for binding [41-43]. However, care must be taken in interpreting the studies with neutralizing antibodies as the HPV virions undergo conformational changes during the infectious process that appear to alter access of L2 to the capsid surface. Further, these conformational shifts might also be triggered upon binding of mature virions to the surface of microtiter plates for ELISA-type studies. In conclusion, it is still controversial which regions of L2 are actually exposed on the capsid surface of fully mature virions in solution. For example, the epitope in HPV16 L2 17-36 recognized by the RG-1 monoclonal antibody is accessible on virions bound to ELISA plates, but not mature virions in solution [44]. Rather, it becomes revealed after binding to the extracellular matrix and furin cleavage of L2 [45]. However, other studies indicate that L2 residues 13-31 and 100-120 are constitutively exposed on the capsid surface [42, 46]. Thus much of L2 is buried below the capsid surface of mature virions, but certain amino-terminal regions of L2 can possibly be

exposed on the cell surface during the early events of viral infection [22] (see section on viral host cell entry for details).

Unfortunately, no X-ray crystallographic structures are available for capsids containing L2. Several efforts at high resolution three dimensional image reconstruction of cryo-electron micrographs of native BPV1, HPV1 or CRPV virions, HPV1 or HPV16 VLPs have attempted to visualize L2 in the capsid [22, 30, 32, 47-49]. However, most studies failed to visualize L2, possibly reflecting inadequate incorporation into the capsid, degradation, disorder or lack of symmetry. A study by Trus *et al* detected some protein density at the center of the capsomers at the 5-fold axes of symmetry (pentameric capsomers) of native BPV1 virions, but not the hexameric capsomers, that might be L2. However protein density associated with L2 could not be definitely assigned and the ratio of L1:L2 in BPV1 virions used in this study was ~30:1 [32]. However, in a more recent study comparing the structure of HPV16 L1 only versus L1+L2 particles in which the ratio of L1:L2 was driven close to 5:1, protein density was observed at the base of the axial lumen of all capsomers that was absent in L1 only particles (Figures 2A-C). This is consistent with the notion that much of L2 is buried below the capsid surface, although little of L2 was visualized as the density corresponded with only ~12kDa of its mass [22, 30].

### **L2 facilitates genome encapsidation**

DNA-binding activity *in vitro* has been displayed by PV L1 and L2 using BPV1, HPV6b, 11 and 16.[50-52] This in turn, suggests the capsid proteins aid in viral genome encapsidation during viral assembly. While no sequence-specific binding to DNA has been documented for L1 or L2, putative DNA-binding regions have been recognized as conserved short sequences of positively charged basic residues. Such regions can be found in the carboxyl terminus of L1 and in both the amino and carboxyl termini of L2. These regions on L1 in HPV33 [51] and BVP 1 L2 [53, 54] respectively have also been reported to be required for efficient viral DNA encapsidation, although with respect to L2 the effect may be less pronounced for

HPV16, HPV31 [17, 55] and HPV33 [56]. In addition, these basic residues at both the N and C terminus of L2 can also function as nuclear localization signals (NLS) although it was later reported that only the amino-terminus can bind to DNA *in vitro* [23, 57-60].

Interestingly, there seems to be no correlation between binding activity and encapsidation efficiency as studies on BPV1 showed deletion of either terminus from BPV1 L2 has no impact upon BPV1 genome encapsidation [61]. Taken together, it seems that there is no specific HPV capsid protein-DNA interaction. Rather, given that it has been shown that L2 can bring components of the PV virion to specific locations in the nucleus, it is more attractive to hypothesize that via an undocumented mechanism which concentrates PV L1, L2 and viral genome, such conditions instead allow L1 and/or L2 to facilitate encapsidation and assembly by binding to the histones around which the genome is wound rather than via a specific mechanism that directs interaction with the DNA [62, 63].

### **L2 and host cell entry**

There are currently two competing models of papillomavirus infection events prior to cell entry [45, 64]. However both agree that L2 starts predominantly buried within the capsid and its exposure requires a conformational change induced by capsid binding to heparan sulfate proteoglycans that is anchored in the extracellular matrix *in vitro* or the basement membrane *in vivo*, or possibly in solution. This binding induces changes in the conformation of the capsid such that the very amino terminus of L2 extrudes to the capsid surface and becomes susceptible to cleavage by the pro-convertase enzyme furin in the extracellular milieu, thus removing one of the putative NLS [45, 46, 65-67]. Analysis of the N-terminus of all PV types reveals that the consensus furin cleavage motif site (R-X-K/R-R) (Figure 3B) is conserved at around amino acids 9-12 [46]. Numerous PV types exhibit reduced infectivity upon exposure to furin inhibitors both *in vitro* and *in vivo*, and furin deficient cells are not infected unless complemented with the a furin expression vector or extracellular addition of furin. Point mutation of the furin cleavage site also rendered virions non-infectious [46]. Thus cleavage of

L2 by furin is required for infection and results in further capsid conformational changes. The virus is subsequently internalized via an undefined secondary receptor found on the host cell basal keratinocyte (Figure 3A). Importantly, furin cleavage also exposes a broadly neutralizing epitope (amino acid region 17-36) which was first characterized using the RG-1 monoclonal antibody [68].

Richards *et al* showed L2 with point mutation of L2 preventing furin cleavage did not affect PsV production. These mutant PsV had typical levels of L2 and were able to encapsidate the reporter genome normally. These mutant capsids were also able to bind to the cell surface, enter the cell, traffic through the endosomes and uncoat normally. However, the mutant L2 and reporter genome were unable to leave the endosome even after 24 hours post-infection while wildtype L2 and reporter genome accumulated in the nucleus. Endosomal retention was also observed in infection studies with wildtype PsV incubated with a furin inhibitor. These findings suggest that furin cleavage of L2 is important for the exit of the L2/genome complex from the endosomal compartment [46, 66].

There is no clear difference in the initial internalization of L1 VLPs and L1/L2 VLPs visualized by fluorescence microscopy. This suggests that it is either L1 that binds to the secondary unknown entry receptor or these particles are able to internalize but via subtly distinct pathways that are not currently discernable by fluorescence microscopy. Assuming the latter, it has been proposed that furin cleavage exposes on the surface of the capsid a previously buried region of L2 that can bind a currently undefined epithelial cell surface entry receptor, and that it is this L2-receptor interaction facilitates viral entry along the true infectious pathway. Studies have suggested two candidate sites on L2 for binding to a putative entry receptor; one coincident with the RG-1 epitope (13-31), and another at a region on the surface exposed region of L2 (108-120) that is recognized by another neutralizing antibody [42, 69]. The 13-31 region binds to cell surfaces and contains several residues critical for infection by HPV16 [42], notably the previously mentioned two cysteines residues that are

completely conserved [36, 37]. The L2 108-126 region, which contains a neutralizing epitope, can also bind to epithelial cells and exhibits sequence conservation [43, 70]. Interestingly, pre-incubation of 108-126 L2 peptide with cultured cells reduced PsV infectivity by 60% compared to control peptides [71]. Further, annexin A2 heterotetramers (A2t) on epithelial cell surfaces were recently found to interact with this region and they were proposed as a putative candidate for L2 receptor binding [71]. In summary, furin cleavage of L2 is both a critical and universal event in PV infection and it presumably renders L2 able to perform other roles further downstream of the infectious process. Whether L2 is involved in the initial uptake (i.e binding to the secondary receptor) remains a controversial topic and is an active area of both debate and PV research.

### **L2 and vesicular trafficking of papillomavirus**

For successful infection, PV needs to enter the host cell, cross the cytoplasm and transport the viral genome to the nucleus. With respect to entry, this is actually a two-step process involving viral entry and subsequently, viral movement into the appropriate organelles. PV apparently can utilize several different cellular pathways for internalization, although one particular pathway might be dominant and the virus is then able to exploit alternative pathways should the typical pathway be blocked. It should be noted that the typical entry route may vary between the cell line/type and the particular PV genotype studied ([72, 73]). As mentioned earlier, there has been no discernable difference in uptake mechanisms in epithelial cells with respect to L1-VLPs and L1/L2-VLPs with current imaging techniques. However, subtle differences were noted when studying PV uptake using immune cells. For example, L1-VLPs enter cells via either clathrin-mediated or caveolae-dependent mechanisms [74, 75], whereas L1/L2 VLPs were taken up by clathrin- and caveolae-independent mechanisms, suggesting that L2 may impact the uptake pathway. Interestingly while Langerhans cells that are exposed to L1 VLPs functionally matured and L1/L2 VLPs lacked this effect. Fahey et al suggest that L2 may play a role in immune escape by re-routing uptake

[76]. However, inclusion of L2 in VLPs did not impact their activation of bone marrow-derived dendritic cells [77].

In the case of infection of epithelial cells, regardless of the mode of internalization, consistent findings suggest that following cell entry, papillomavirus enters the early endosome and transitions to the late endosome in an acidification-dependent process. Interestingly, the virions have recently been found to pass via the *trans*-golgi network, wherein L1 is retained. Entry to the golgi required furin cleavage of L2, and golgi trafficking mediated by Rab 9a and Rab7b [78]. Other organelles have also been implicated in the trafficking process, notably caveosomes and the endoplasmic reticulum for BPV1, HPV31 and HPV16 [79-83]. Virions that gain entry to the cytoplasm by an unknown and possibly L2-dependent mechanism have also been visualized by transmission electron microscopy, but L1 does not make it to the nucleus [42, 84]. Numerous host cell proteins have also been identified as interacting partners with L2 and have been suggested to facilitate entry and trafficking by various mechanisms to the nucleus. These details will be discussed in the next few sections.

The first example is the chaperon protein cyclophilin B (CyPB). Cyclophilins are peptidyl-prolyl cis/trans isomerases, and it has been shown in other viruses such as HIV whereby CyPA binds via the HIV capsid protein motif 85-PXXXGPXXP-93, which is a similar proline motif those of to L1 interacting motif on L2 [85]. In a similar fashion, CyPB binds to L2 amino acid region 90-110 (now termed the CyPB binding site) and it was thought initially that CyPB-binding merely facilitates the cell-surface exposure of L2 N-termini for furin cleavage and aid subsequent cell internalization (Figure 3A). The authors of the study however noted that if CyPB was inhibited, non-infectious HPV16 PsV internalization could still occur. In addition, PsVs with mutated CyPB motifs could also internalize into the cell independently of CyPB but were still sensitive to CyPB inhibitors [85]. This suggests that CyPB plays 2 distinct roles in HPV infection. Recently, the second role was elucidated in a study whereby it was shown that CyPB aids in the uncoating of HPV capsid in the late endosome. Specifically,

CyPB causes the dissociation of the viral capsid proteins L1 and L2 complexed with the viral DNA into a separate endocytic compartments [86].

Another host protein that has been recently found to be important for virion trafficking is the cytosolic adaptor protein Sortin Nexin 17 (SNX17). SNX17 is an important player in endosomal recycling and can be found in early endosomes and recycling tubules. Key substrates of SNX17 are typically transmembrane cargo proteins with a NPxY motif at their cytosolic tails. Interestingly, studies by the Banks group showed that numerous PV types have a highly conserved NPxY motif at around amino acid region 245-257 of L2 that binds to SNX17. They also showed that knockdown of SNX17 dramatically reduced HPV PsV infectivity. Conversely, over-expression of SNX17 increased infectivity in a dosage-dependent manner. Mutation of the NPxY region reduced viral infectivity suggesting that there is a critical SNX17-L2 interaction. This interaction may prevent premature lysosomal degradation of the virus as NPxY mutant virions experience premature lysosomal degradation and the L2-vDNA complex does not escape the endosome to travel to the nucleus [87, 88].

Lastly, another possible candidate put forward as aiding in PV transport is the tSNARE syntaxin 18 protein [80, 82]. Syntaxin 18 binds to L2, and L2 residues 41-45 are critical for this interaction. Mutation of L2 41-45 in the context of BPV1 eliminated infectivity without compromising encapsidation [80]. Syntaxin 18 is an ER resident protein and overexpression of a dominant negative inhibitor blocked both ER trafficking and BPV1 PsV infection. This suggests a distinct route of trafficking for BPV1.

### **L2 and vesicular escape during infection**

Following virion dissociation, the viral DNA (vDNA) must escape the vesicular compartment to be able to travel to the host cell nucleus. Studies using labeled PsV genome and L2 antibodies have suggested that L2 in complex with HPV vDNA somehow exits the late endosome to carry out this process. The information on the mechanism of HPV endosomal



escape is an area of active investigation. Kamper et al showed that HPV33 L2 C-terminus has a 23 amino acid region that contains adjacent hydrophobic and basic clusters of amino acids. The authors showed that full length L2 with this region or this region fused to GFP was able to integrate into cellular membranes. The deletion or mutation of this region also abrogated viral infectivity and consequently the viral genome together with L2 was retained in the late endosome suggesting that this region could potentially act as a membrane destabilizing peptide for L2/vDNA endosomal escape [89].

Bronnimann et al recently described another region in L2 that potentially could also aid endosome escape. They found that L2 has a transmembrane-like domain region at its N-terminus spanning residues 45-67, that includes several highly conserved GxxxG motifs (Figure 4). Mutagenesis of some of these GxxxG motifs resulted in endosomal retention of L2 suggesting the importance of this region in infection. The authors also noted that the predicted structure of this domain is alpha-helical in lipid environments and that these motifs lie on two opposite faces of this helix (Figure 4B). Interestingly, based upon some in vitro studies, these faces could self-associate within biological membranes using the GxxxG motif. Furthermore, they provide evidence that multiple L2 molecules can associate either homo- or heterotypically via these GxxxG motifs to potentially form a higher order structure that could synergistically facilitate endosomal penetration [90].

These findings do not rule out other host cell protein interactions with this TM domain that could aid endosome escape. Indeed, we have recently shown that gamma-secretase (GS) is required for PV infection. Interestingly, GS inhibitors prevent HPV16 PsV infection and result in retention of L2/genome complex in the late endosome [91]. This suggests that GS facilitates egress from the late endosome, an L2-dependent process, but its key substrate in this process is not known. GS cleaves substrates at their transmembrane domain and the above-mentioned studies suggest that L2 has such a domain indicating a possible interaction. Indeed substrates of GS are also typically cut with a pro-protein convertase at a position less

than 30 residues from the transmembrane domain. Interestingly, L2 is cleaved by the proprotein convertase furin at approximately amino acid region 9-12 which is ~30 residues from the putative transmembrane domain. As mentioned, inhibition of furin and GS cleavage both result in endosomal retention of L2 and the viral genome, suggesting these processes may be functionally related.

### **L2 and the cytoskeleton**

Upon endosome escape, the L2/vDNA complex may utilize the cytoskeleton to traverse the cytoplasm. L2 is able to interact with beta-actin at a conserved site found at residues 25-45 [92], but the locomotive mechanism involving beta-actin was not defined. Florin et al describe the binding of L2 with dynein motors via its C-terminal 40 amino acids. The authors demonstrated via immunofluorescence and co-immunoprecipitation that L2 via binding to the motor protein dynein could interact with the actin microtubule network and conduct minus-end-directed transport of L2/vDNA to the nucleus [62]. In a subsequent study, the same authors further substantiated this using a combination of two-hybrid assays and immunofluorescence to reveal DYNLT1 and DYNLT3 (Dynein light chain 1 and 3) as the main components that work with L2 for microtubule transport [93].

### **Nuclear entry by L2**

Multiple possible interacting partners of L2 have now been identified as aiding traversal of the cytoplasm and vesicular trafficking. Importantly the mechanism of nuclear entry by L2 differs by stage of the virus life cycle, i.e. viral entry into the nucleus during the establishment of initial infection versus during virion production. With respect to the former, it is well established that L2 is required to go to the nucleus with the vDNA to enact initial infection [63, 94]. Initially, it was assumed that the L2-vDNA complex entered the nucleus via nuclear pore complexes via the previously mentioned NLS on both the N- and C-termini of L2. However, the N-terminus NLS is located upstream of the furin cleavage site and thus might not be present after entering the cell during infection. Similarly, the C-terminus NLS is near

the membrane destabilizing peptide and may also be a DNA-binding domain, potentially blocking this NLS. In light of this, Pyeon et al showed in a surprising study that the L2-genome complex enters the nucleus, not via the nuclear pores during interphase, but rather requires entry to mitosis associated with the breakdown of the nuclear envelope [95]. Mamoor et al also described an arginine-rich nuclear retention signal in L2 has been described in HPV16 L2, comprising residues 296-SRRTGIRYSRIGNKQTLRTRS-316 that is required for infection but not virion assembly. It is possible that this region allows for the association of L2 and the genome complex with the nuclear matrix during metaphase. In the same study, a leucine-rich nuclear export signal (NES) was also described at the C-terminus of HPV16 L2, encompassing residues from 462-LPYFFSDVSL-471. This leucine-rich region on L2 along with some deletion mutants of the same region was subsequently fused to GFP and was found to exert nuclear export in a CRM1 (Exportin)-dependent manner. At present, the role of this NES is not clear but it does not appear to be critical for either virion assembly or infection [96].

While the roles of the nuclear localization signals (NLS) at both its N- and C-termini are unclear with respect to initiation of infection, these regions can independently interact with karyopherins, specifically  $\text{kap}\alpha\beta 1$ ,  $\text{kap}\beta 2$  and  $\text{kap}\beta 3$ , for entry into the nucleus via the nuclear pore complex after the initial ribosomal synthesis of L2. It is interesting to note BPV L2 interacts with  $\text{kap}\alpha\beta 1$  only which suggests that the nuclear import pathways are different for different PV types [59, 60, 97, 98]. Heat shock cognate protein 70 (Hsc70) can also assist in transporting newly synthesized L2 back into the nucleus via forming an active complex with L2. Hsc70 is necessary for L2 transport to the nucleus as depletion of Hsc70 resulted in L2 accumulation in the cytoplasm. Apart from nuclear transport and entry, Hsc70 may facilitate viral assembly possibly by maintaining L2 in a specific state of folding that is optimal for interaction with the L1-capsomers. This role was proposed upon finding that Hsc70 could be co-sedimented with L1/L2-VLPs but not L1-VLPs suggesting that Hsc70 is associated with L2 upon its integration into VLPs. Hsc70 could not be detected with PsV

suggesting that it can be eliminated by an undefined mechanism during the process of DNA encapsidation [99, 100].

### **Nuclear activities of L2**

There is a motif around amino acid region 390-420 that targets L2 to the ND10 subdomains of the nucleus (also known as PML-oncogenic domain/POD/PML bodies and defined by co-localization with PML) [63, 99, 101-103]. HPV E1 and E2 can also associate with ND-10 domains during viral DNA synthesis [104]. Taken together, it was proposed that L2 may recruit other PV viral components to this area possibly to facilitate structural assembly and/or vegetative replication of the PV genome [51, 63, 105, 106]. Whereas BPV1 L1, E1 and E2 exhibit diffuse nuclear staining when produced individually using recombinant Semliki Forest Virus vectors, both BPV and HPV L2 when overexpressed would cause both L1 and E2, but not E1, to co-localize in a specific nuclear region known as the ND-10 domain [63]. Given the importance of L2 in assembly of BPV virions (and to a smaller extent HPV virions), these findings suggested that L2 might act to organize/co-localize the virion components adjacent to ND-10. However, Buck et al found no requirement for E1 or E2 in the assembly of HPV pseudovirions [17], although this production system may not fully mimic vegetative replication and virion assembly occurring in differentiated epithelium.

It has also been suggested that ND-10 localization is actually non-physiological and that the L2 accumulation observed is due to the over-expression system and/or misfolding [107]. However, Day et al also observed that establishment of a papillomavirus infection and viral transcription is reduced 10-fold in the absence of PML protein (a critical structural component of ND-10), and that upon infection L2 traffics to ND-10 in association with the viral genome [106]. These findings once again point to the importance of the ND-10 localization of L2. However, here it is proposed that the organization of viral components at ND-10 by L2 may facilitate the early burst of transcription and replication events at the initiation of infection.

Studies with HPV PsV also show that they bring their encapsidated reporter DNA vectors to ND-10 and efficiently initiate reporter gene expression and vector replication via SV40 T antigen at low MOIs. While this implies that the interaction between L2 and the encapsidated DNA is not sequence specific, and initiation of infection does not require direct interaction between L2 and E2, it does not rule out a role for L2-E2 interactions in the initiation of infection by authentic PV virions. Indeed, several studies suggest interactions between L2 and E2. It was found that the amino terminal residues 1-50 of L2 are responsible for binding to E2. However, L2 residues 301-400 are also required for the down-regulation of E2-dependent transcriptional activation. L2 does not reduce transcriptional activation by either lowering E2 protein levels via reduction of mRNA levels or by enhancing its proteasomal degradation [108]. Heino et al also found that L2 inhibits the transcriptional activation function of E2 transcriptional transactivator (E2TA), but not its capacity to support viral DNA replication. Interesting, L2 can bind to and bring the E2-TR transcriptional repressor form to ND-10, as it binds to two separate domains of E2 [105]. Taken together, these findings indicate a possible role for L2 in early viral transcription as infection is initiated.

L2 also causes changes in the ND-10 environment; overexpression of HPV33 L2 induces the recruitment of Daxx, a nuclear transcriptional repressor protein that represses transcriptional activators, to ND-10. The interaction of Daxx with L2 was further confirmed via co-immunoprecipitation with L2. Furthermore, overexpression of L2 results in the expulsion from ND-10 and subsequent degradation of the transcriptional activator, SP100. Interestingly, proteasome inhibitors prevent the accumulation of L2 in the nucleus under these conditions and the accumulation of L1 at ND-10 with L2 only occurred after the exit of SP100. This adds to the speculation that the accumulation of L2 and Daxx followed by the loss of SP100 potentially could result in a major reorganization of the ND-10 environment to facilitate the assembly of virions [102, 103]. The domain of HPV 33 L2 (390-420) that was required for ND-10 localization, was also required for the loss of SP100 and accumulation of Daxx. It was also suggested that interaction with Daxx may be also be the driving force for the localization

of L2 into ND-10 [102, 103]. However, it is important to recognize the caveat that these data were derived using an over-expression system [101, 107].

Given L2's interaction with E2 and inhibition of E2-dependent transcriptional activity, it seems plausible that L2 has a role also in transcriptional regulation. Indeed, a number of relevant L2-interacting partners have been identified using yeast 2-hybrid approaches.

Gornemann *et al* detected four nuclear body-associated proteins that could interact with the L2 of different HPV types and co-localize in the ND-10 domains in some cases. PATZ is a transcription factor which was found to co-localize in discrete subnuclear domains and to bind with L2. Interestingly, PATZ is a zinc finger family transcriptional regulator and it was postulated that L2-PATZ interactions might play a role in gene regulation and cell differentiation during papilloma formation. Other interesting potential interactions were identified, including TIP60, TIN-Ag-RP and PLINP. However, all these identified interactions require further study to understand their function [109]. In a more recent study using the 2-hybrid approach again, the transcription factors TBX2 and 3 were identified as interacting partners of L2 at its C-terminus using HPV11, 16 and 18. The study also found both TBX2 and TBX3 repress viral transcription from the viral LCR of several HPV types and this repression was enhanced when L2 was over-expressed. In particular, the levels of early genes such as E6 and E7 were reduced in HeLa cells when TBX2 was increased.

Interaction of TBX2 with the LCR was confirmed by CHIP analysis, and L2 seems to be a key player in stabilizing this interaction. TBX2/3 co-localization with L2 can also occur either in the ND-10 domains or throughout the nuclei depending on the distribution of L2. It is important to recognize that L2 is usually diffused throughout the nucleus in the upper stratified layers of CIN1/2 lesions [110], and it is possible that L2-TBX2/3 interactions here serve to inhibit early viral gene expression and aid the transition to viral assembly during infection [111].

## **L2 Immunology**

Two L1 VLP vaccines (GlaxoSmithKline's Cervarix® and Merck's Gardasil®) have been approved by the FDA for the prevention of HPV-associated cervical neoplasia. Cervarix® contains HPV16 and 18 L1 VLPs, whereas Gardasil is a mixture of these two types as well as L1 VLPs of low risk types HPV6 and 11 that cause genital warts. While both vaccines showed high efficacy in preventing HPV infection in many human clinical trials [112-116], the protection these vaccines confer is type-restricted. Given this limitation, second generation HPV vaccines are being developed. Indeed, Merck is currently testing a nonavalent L1-VLP HPV vaccine targeting 7 high risk HPV types to broaden protection among the types most commonly detected in cervical cancer. However, complex formulation is expensive which could drive up costs and thus indirectly discourage vaccine usage in low resource settings where a broadly protective HPV vaccine is most needed [117].

Alternatively, the HPV minor capsid protein L2 is an interesting candidate that may address the aforementioned requirements of cost and broad coverage. L2 can be expressed as a single antigen in bacteria and this could potentially bring down production costs. More significantly, vaccination studies with L2 in animal challenge models showed the production of antibodies neutralizing for a wide spectrum of PV types, and similarly broad protection from experimental viral challenges [69, 118-123]. Passive transfer of L2-specific neutralizing antibodies is sufficient to mediate protection of mice from cutaneous or vaginal challenge with HPV pseudovirions [7, 68, 120, 124]. While some variation in these neutralizing epitopes is apparent [125], sequence comparison at the N-terminus of L2 reveals high conservation across PV types (Figure 5), including regions that are recognized by neutralizing monoclonal antibodies (Figure 1 and Table 1) [41, 126]. The reason for sequence conservation in these L2 epitopes which is potentially detrimental to the virus has been debated. Several explanations have been put forward as to why the neutralizing epitopes are conserved in L2, but not L1. Firstly, L2 is hidden in the virus capsid unlike the surface loops of L1 that form the immunodominant neutralizing epitopes of VLPs. This could explain the lack of evolutionary pressure for these epitopes drift in sequence [61]. Secondly, the

conservation of the L2 neutralizing epitopes also suggests an evolutionary constraint that may reflect a role in this region that possibly binds to a cellular entry factor during infection [42, 69, 71].

Unfortunately, L2 provided no discernable benefit in the context of L1/L2 VLPs [127, 128], and the immune response produced to vaccination with L1/L2 VLPs was found to be predominantly an anti-L1 response [129]. This skewed response has not been fully understood but has been attributed to the overall number of L1 (which have numerous immunogenic epitopes also) compared to L2 in a single virus capsid, and distant spacing of L2 compared to L1, and possibly low occupancy of L2 in L1/L2 VLPs. Further, L2 is buried in the PV capsid and thus possibly hidden from or only exposed transiently to B cells. The challenge ahead for L2-based vaccines is to find methods to expose L2 to the immune system to induce and maintain protective levels of antibodies to these L2 epitopes for many decades. Many methods have been utilized with varying success at the preclinical setting and this will be discussed subsequently.

### **Conclusions**

It is clear that L2 has complex and multifunctional roles in the biology of all PVs, notably in virion assembly and early events of infection, and has potential as a vaccine antigen. A recurring theme in our discussion of L2 biology is that while many functional domains and numerous host-cell protein interacting partners are now recognized (Table 2), the detailed mechanism of their action remains controversial or unclear in many cases. The challenges ahead for PV L2-based research are to obtain an atomic structure of L2, and to decipher clearly how host proteins that interact with L2 impact the biology of PV. The PaVE website will be a valuable tool to collate and disseminate these findings.





## PAPILLOMAVIRUS L2 PROTEIN

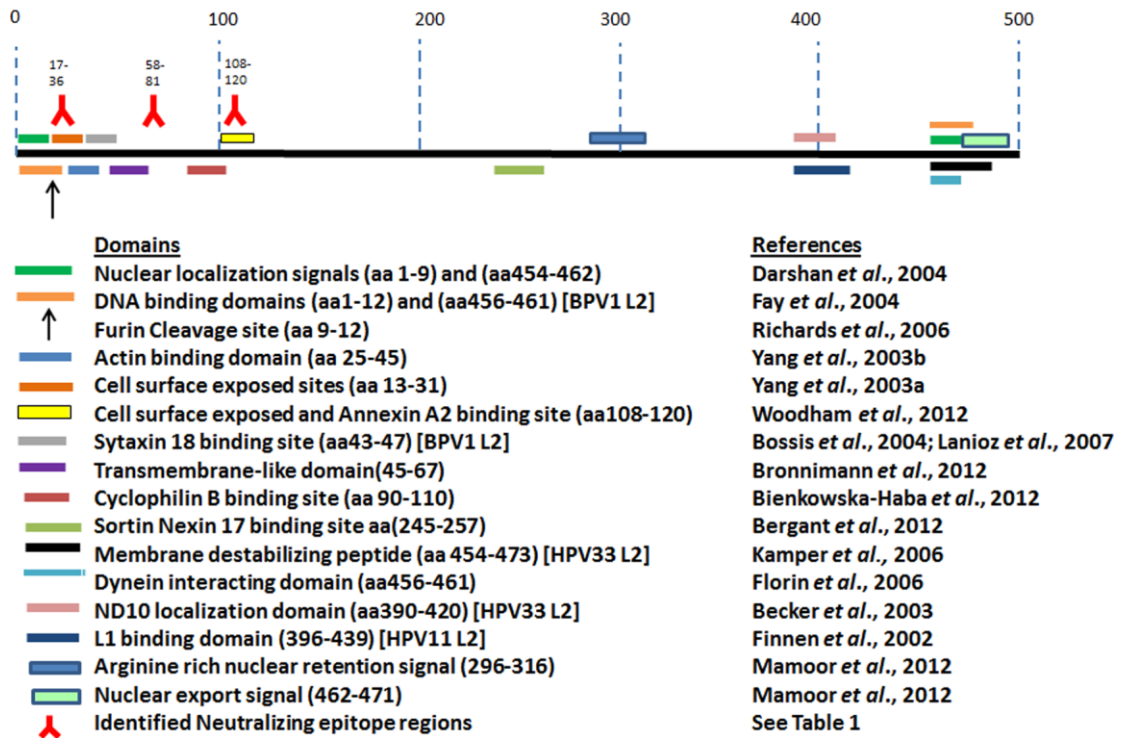


Figure 1. **Diagram of known neutralizing epitope regions and protein interaction domains of HPV16 L2. Regions that were discovered using other PV L2 types are indicated.** Diagram was adapted and updated from [130]. See Table 1 for a full list of neutralizing antibodies..

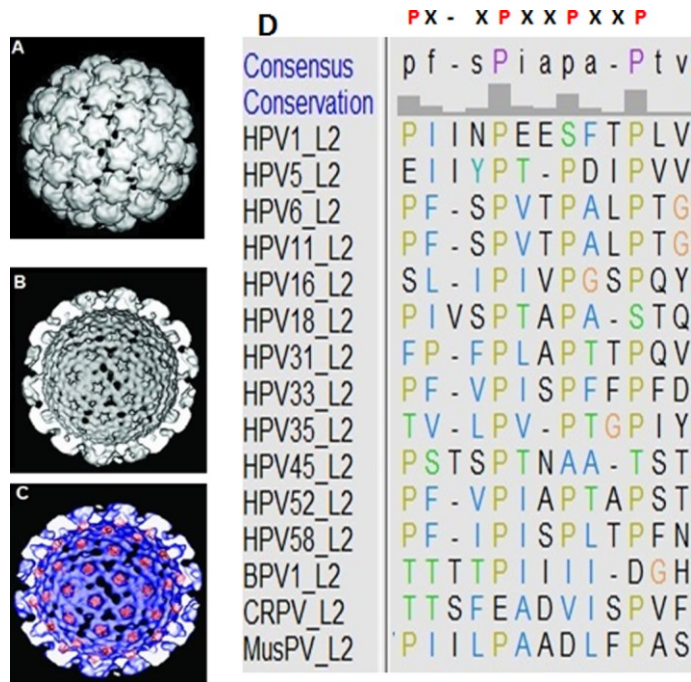


Figure 2. **Arrangement of L1 and L2 in HPV16 capsids.** 3D reconstructions derived by comparing cryo-electron micrograph images of HPV16 capsids made of L1 + L2 (A). Interior of L1+L2 capsid without DNA and histones (B). Arrangement of L2 density (red) areas superimposed on the interior view of L1 only capsid (in blue) (C). Clustal analysis of L1 binding domain of L2 showing several conserved proline (PxxP) motifs (D). Clustal Analysis was performed with the UCSF Chimera package. Images were provided by courtesy of both Christopher Buck and Benes Trus, NCI.

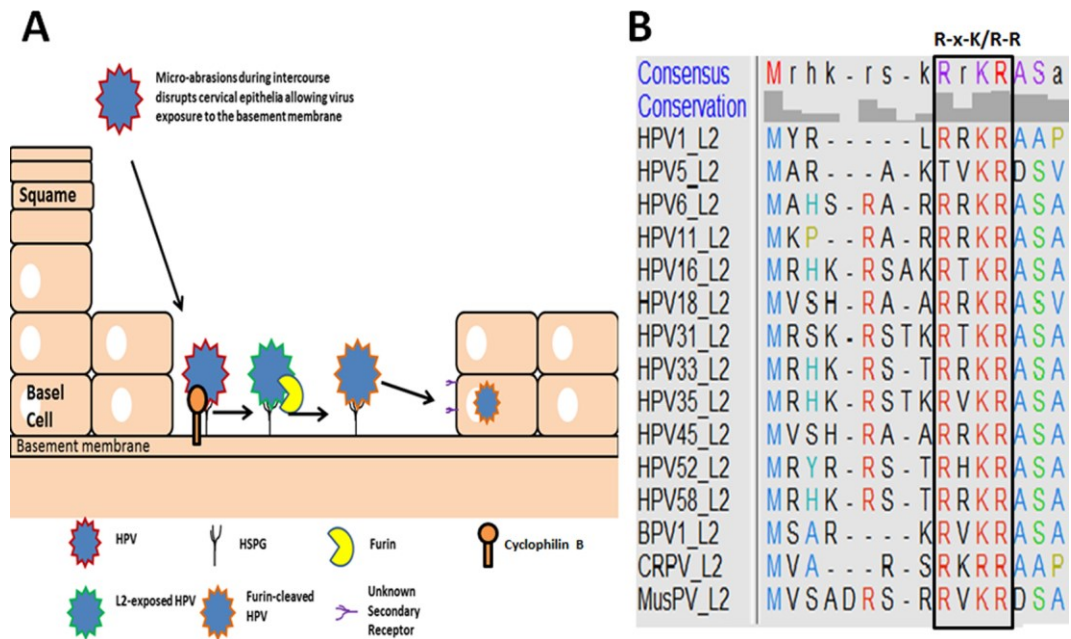


Figure 3. **A model depiction of the early events of PV infection in the cervical epithelium in vivo.** Exposure of the basement membrane by micro trauma allows PV to bind to Heparan Sulfate Proteoglycans (HSPG). Binding of PV to HSPG and subsequent interaction with host cell protein Cyclophilin B causes a conformational change in capsid structure resulting in the exposure of the N-terminus of L2. L2 has a furin cleavage motif and is cleaved by furin. Subsequently, L2 is internalized into the basal cells (3A). Clustal analysis of the furin cleavage motif, R-x-K/R-R (boxed in black) on a variety of HPV and other commonly studied animal PV L2 (3B). Clustal Analysis was performed with the UCSF Chimera package

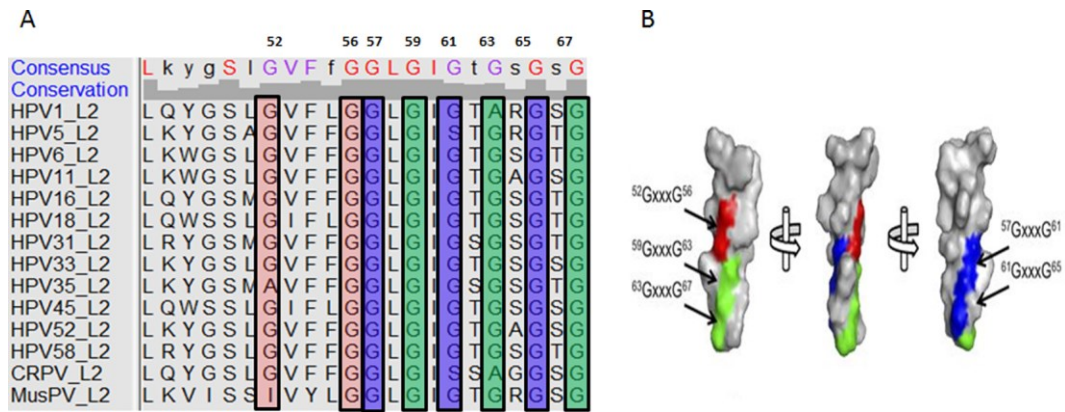


Figure 4. **The putative transmembrane domain of L2.** Clustal analysis of the transmembrane-like (TM-like) domain in L2 of fourteen PVs (A). Alpha helical modeling of the TM domains with the colors indicating the conserved GXXXG domains on each face (B). Images were provided by courtesy of Samuel Campos, University of Arizona. Clustal Analysis was performed with the UCSF Chimera package

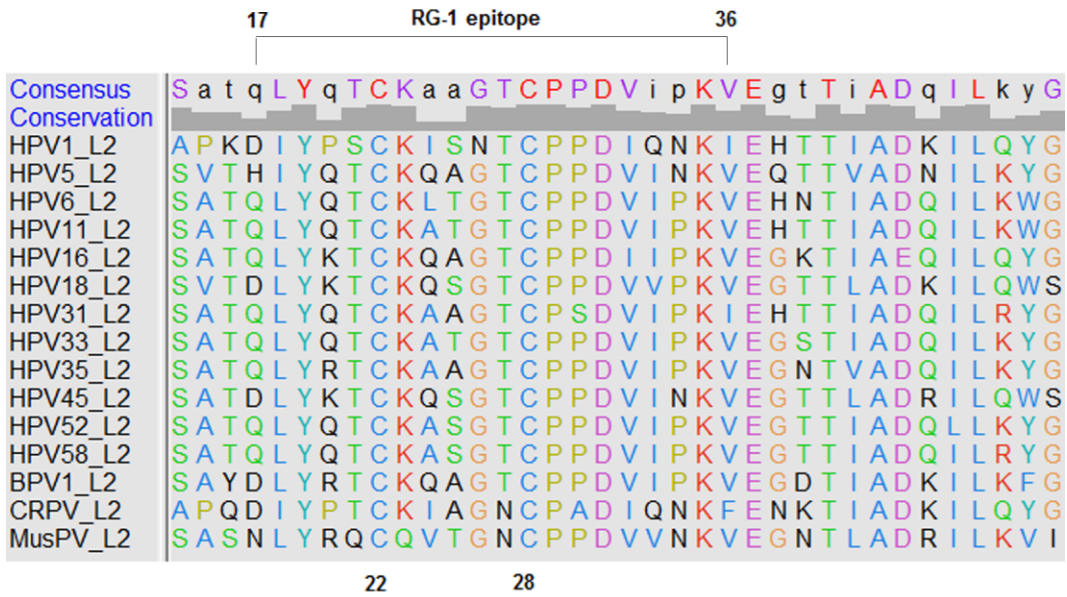


Figure 5. Strong sequence conservation of 17-36aa region (commonly known as the RG-1 epitope) among different HPV types. Note the two cysteine residues (C22 and C28) in this region are important for infection and neutralization. The exception to this (with regards to infection upon mutation of cysteine) is in BPV1. Clustal Analysis was performed with the UCSF Chimera package.

Table 1. **Published HPV16 L2-specific neutralizing and cross-neutralizing antibodies.**

<b>Monoclonal antibody designation</b>	<b>Epitope region in HPV16 L2</b>	<b>Cross Neutralizing ability</b>	<b>Isotype</b>	<b>References</b>
RG-1	17-36	Yes	IgG1	[68]
WW1	17-36	Yes	IgG2a	[131]Manuscript in preparation
MAb24B	58-64	Yes	IgG2b	[132]
MAb13B	64-73	Yes	IgG1	
K4L2 <sub>20-38</sub>	21-30	Yes	IgG2b	[41]
K18L2 <sub>20-38</sub>	22-30	Yes	IgG1	
K8L2 <sub>28-42</sub>	32-39	16 only	IgG1	
K1L2 <sub>64-81</sub>	73-79	16 only	IgG1	
MAb5	108-120	Yes	IgG2a	[70]
MAb13	108-120	Yes	IgG3	
16L2.4B4	108-120	Yes	Undefined	[71]

Table 2. Published list of known protein interactions with PV L2.

<b>Stage of PV Virus life cycle</b>	<b>Protein that interacts with L2</b>	<b>Interacting region on L2</b>	<b>Does the protein recognize a specific motif/ consensus sequence on L2?</b>	<b>Purpose of interaction</b>	<b>Reference</b>
<b>Prior to infectious cell entry</b>	Furin	9-12	R-X-K/R-R	Cleavage of L2	Richards et al., 2006
	Cyclophilin B (CyPB)	90-110	N/A	Assist in capsid conformational change for secondary receptor uptake	Bienkowska-Haba et al., 2009
	Annexin A2 Heterotetramer	108-120	Neutralizing epitope region 108-120	Putative secondary receptor	Woodham et al., 2012
<b>Vesicular trafficking &amp; Endosome escape</b>	Cyclophilin B (CyPB)	90-110	N/A	Assist in capsid disassembly in the late endosome	Bienkowska-Haba et al., 2012
	Gamma-secretase (GS)	Unknown	Unknown		Karanam et al. 200



	Sortin Nexin-17 (SNX17)	245-257	F/YxNPxF/Y	Facilitates L2/vDNA endosomal escape	Bergant Marusic et al., 2012  Bergant and Banks et al., 2013
<b>Nuclear transport</b>	Syntaxin 18 (BPV1 only)	43-47	DQ/KILQ/K	Transport of L2 toward nucleus	Bossis et al. 2005  Laniosz et al., 2007a
	Heat Shock cognate protein 70 (Hsc70)	Unknown	N/A		Florin et al., 2004
	Beta-Actin	25-45	N/A		Yang et al., 2003b
	Dynein motor proteins (DYNLT1 & 3)	456-460, 457-461 (HPV 16 L2)	R/KR/KXXR/K		Florin et al., 2006  Schneider et al., 2011

	Karyopherins (Kap $\beta_2$ and Kap $\beta_3$ )	1-9 or 454-462 (NLS signals at the amino and carboxyl termini respectively)	N/A		Bordeux et al.,2006; Darshan et al.,2004; Klucevsek et al., 20Se06; Fay et al., 2004; Sun et al., 1995
<b>Viral gene regulation, morphogenesis and assembly</b>	ND10 domain and binding to Daxx	390-420	N/A	Recruits other viral proteins and viral DNA to initiate viral gene transcription or assembly?	Becker et al., 2003  Florin et al.,2002b
	PV E2	1-50 and 301-400	N/A	L2 brings E2 to the ND10 domains (possibly to initiate viral gene transcription or replication)	Okoye et al., 2005  Heino et al., 2000
	PV L1	412-455	PXXP	Viral capsid assembly	Finnen et

					al.,2003
					Lowe et al.,2005
	TBX2 and TBX3	L2 C-terminus region	N/A	L2 brings TBX2/3 to the viral genome's LCR where TBX2/3 then represses of viral gene transcription	Roanowski et al., 2013
<b>Other known interaction protein partners but with undefined function</b>	<ul style="list-style-type: none"> <li>• PATZ</li> <li>• TIP60</li> <li>• TIN-AG-RP</li> <li>• PLINP</li> </ul>	Unknown	Unknown	Unknown	Gronemann et al., 2002
	SUMO (small ubiquitin-related modifiers)	34-37	PVKE	Unknown	Marusic et al.,2010

### **C) Immuno-prevention of human papillomavirus-associated malignancies**

Cervical cancer remains the third leading cause of cancer in women worldwide and virtually all cases can be attributed to infection via one of fifteen high risk HPVs (hrHPV) [133]. Importantly, significant fractions of other anogenital malignancies (vaginal, vulval, anal and penile) as well as oropharyngeal and oral cancers [134] are also caused by HPV infection, predominantly by the HPV16 genotype. Infections with hrHPV and their associated neoplasia remain highly prevalent and although there are effective screening strategies available for detection of HPV infection at the cervix, importantly, HPV screening strategies are not routinely applied to other anatomic sites.

Since 16-18% of cancer cases globally are caused by infections [21] and preventive vaccination against infectious agents ranks among the most cost-effective of medical interventions, the concept of ‘cancer immuno-prevention’ offers enormous promise to improve human health. Indeed, this promise is being realized with the introduction of preventive vaccines against Hepatitis B (HBV) and HPV, and complements both cervical cytology screening efforts and chemoprevention via drug treatments for *Helicobacter pylori*, Hepatitis C virus, and HIV [133]. While prevention of liver cancers linked to HBV via vaccination has already been demonstrated [135-137], it is too early to see the impact of HPV vaccination on cancer rates although HPV disease rates are clearly dropping [138].

Unfortunately, HPV vaccination uptake has been slow in some developed and many developing countries. The current high cost of the vaccine and need for administration to adolescents have hindered widespread introduction. Motivation for uptake among the general population has also been complicated by perceived concerns for safety, potential for increased promiscuity, lack of efficacy and need, but with continued scientific reporting of the benefits and safety of HPV vaccination, it is hopeful that these concerns will be resolved and implementation will improve.

Here I will discuss the potential of immunization for both prevention and treatment of HPV disease as primary and secondary cancer prevention strategies. It is not possible to cover these areas comprehensively and my intent is only to provide a concise summary of recent advances and areas of promise in immune-prevention of HPV malignancies and how they could interface with current cervical cancer prevention efforts.

### **Etiology of HPV and the early successes of HPV vaccination**

Over 100 different HPV genotypes have been fully sequenced and can be generally divided into cutaneous and mucosal types. While most infections are benign, those caused by a subset (approximately 15) of the mucosal HPV types can progress to malignancy and are considered ‘high risk’ (hrHPV). The hrHPV are the primary etiologic agent of almost all cervical cancers [139-143]. HPV16 [144] and HPV18 [145] are the most studied HR types as they cause ~70% of all cervical cancers [146]. More recently, the link between mucosal HPV and cancers has been expanded to certain subsets of anal, vaginal, vulval, penile and oropharyngeal cancers[140]. HPV16 is also the cause of approximately 90% of HPV-associated cancers at these non-cervical sites [140]. The currently licensed HPV vaccines, Gardasil® (Merck & Co) and Cervarix® (GSK), are based on the major capsid protein L1 which has self-assembled into non-infectious virus-like particles (VLP) [24, 147] and both target HPV16 and HPV18. While the mucosal hrHPV types have received the most attention, the ‘low risk’ mucosal HPV types also produce disease with considerable morbidity, including recalcitrant anogenital warts, and life-threatening laryngeal papillomas (e.g. HPV6 and 11). Gardasil® also prevents infection and disease associated with the two most prevalent types in benign genital warts (90%), HPV6 and HPV11.

Randomized controlled trials for both Gardasil® (FUTURE trials) [148-152] and Cervarix® (PATRICIA and the Costa Rica HPV vaccine trial, CVT) [153-155] examined three immunizations in young women. The vaccines demonstrated high immunogenicity, excellent

safety profiles and showed efficacy in preventing incident vaccine-related HPV infection as well as incident persistent infection related to vaccine HPV types. Several countries have adopted national HPV immunization programs which have begun to bear fruit. For example, in 2007 Australia was one of the first countries to adopt such a campaign with Gardasil® and a significant decline (<1% of women versus 10.5% prior to introduction of vaccines in 2006) in genital wart diagnoses in women [156, 157] as well as reduced cervical abnormalities in teenage girls [158] has been reported. Significant declines in the reporting of genital warts in men were also observed although this finding was attributed to herd-immunity rather than direct vaccination [159].

### **Improving access to current vaccines**

When the HPV vaccine was first introduced, the duration of immunity was also unknown. Therefore pre-teens were considered the optimal population for immunization given the importance of vaccination prior to sexual debut [138]. The targeting of 9-26 year old patients, especially young adolescents however has complicated vaccination because it is infants that traditionally receive the majority of vaccinations. Further, compliance towards 3 doses has been an outstanding issue. In light of this, it is reasonable to consider exploring the safety and immunogenicity of HPV vaccines in infants. Alternatively, co-administration of current HPV vaccines with other childhood combination vaccines against multiple infectious agents should also be considered since this potentially reduces the costs of vaccine administration. Indeed, some HPV co-immunization studies thus far have shown that antibody responses from either vaccine to be non-inferior to when administered alone [160-163].

The cost of the two commercial vaccines has also limited vaccine uptake, especially in the developing world where >80% of cervical cancer cases occur. The vaccines were introduced at \$120 dose, i.e. \$360 total, although recent GAVI pricing of \$4.50/dose for developing countries has been negotiated and is hopefully sustainable [164]. On a national scale, the costs of HPV vaccination provide a significant barrier to mass vaccination programs especially as

they are borne in addition to ongoing costs in cytologic screening programs. It is clearly important to understand how completion of an HPV vaccination regimen impacts a patient's need for cytologic and/or HPV screening. Presently, because the vaccines target only 2/15 oncogenic types, cervical cytology screening continues. However, since HPV vaccination reduces the incidence of high grade CIN, the predictive value and cost-effectiveness of cytologic screening will drop in this population. This issue might potentially be addressed by implementing screening via HPV testing and increasing the screening interval.

Several studies are examining whether fewer doses (2 doses versus 3) can be administered with acceptable protective efficacy and duration [165-171]. Although more independent studies are required to ensure sustained efficacy and examine cross-protection of related types, the data thus far are supportive of a two dose regimen. Such studies have also been examined by health authorities such as the WHO SAGE (Strategic Advisory Group of Experts on immunization) and the overall conclusions were that a 2-dose prime-boost schedule within an interval of 6 months is non-inferior to the standard 3-dose schedule [172] However it is important to note that there were some findings suggesting poorer clinical efficacy with 2 doses which were attributed to a failure in controlling the intervals between the prime and booster immunization. WHO SAGE recommends adolescents within 9-13 years range for the 2-dose regimen and that the interval between the prime and booster shot needs  $\leq 6$  months or else a third boost will be required. There is now considerable interest in the efficacy of a single dose vaccination. Remarkably, in one recent study, a subset of patients that received only one dose of Cervarix® still exhibited detectable antibody responses after four years and no evidence of breakthrough infection [173]. While these findings must be interpreted cautiously, they suggest that a trial to specifically address the efficacy of a single dose may be warranted.

### **Protection against more genotypes**

The current HPV vaccines are not approved for protection against the non-vaccine hrHPV types that can also cause cervical cancer and indeed are currently responsible for ~30% of all cases [174]. Further analysis from both pre-clinical and the HPV vaccine trials showed cross-protection against hrHPV types that are not directly targeted by the current HPV vaccines was observed but was generally partial, limited to a few genotypes and is of unclear duration [175-177]. Therefore, it remains an important goal to extend protection to all hrHPV types without substantially driving up cost of immunization.

Broad protection is especially important for immune compromised individuals who suffer more disease associated with HPV types beyond those targeted by the current vaccines. HIV+ patients have much higher rates of multi-type infections and an increased risk for HPV-associated cancers (e.g. anal cancer in men) [178] despite the introduction of HAART therapy. Linkage studies have also shown a 2-22 fold increase incidence of cervical cancer in HIV+ women compared to HIV- women [179]. While B cell responses are somewhat compromised in HIV+ patients and solid organ transplant recipients (SOTR), vaccination studies in other infectious diseases show they are still capable of generating effective neutralizing antibody responses [180-182]. In light of this, several trials are ongoing to evaluate if this is also true for the current HPV vaccines (reviewed in [182]). In one recently completed trial [183], all HIV-positive women (HAART naïve) were seropositive for HPV16 and 18 antibodies following vaccination with the bi-valent vaccine although titers were overall lower than the healthy control group. The safety profile was also consistent with clinical experience with healthy women. It is worth emphasizing however that certain meta-analysis and population studies indicate that HPV16 infection is under represented in HIV+ patients, suggesting these populations acquire more distinct HPV genotypes or they experience more multi-type infections. While this further complicates current HPV vaccine uptake as well as policy making decision since not all types are targeted by the current HPV



vaccines, the findings also support the efforts to make a more broadly protective HPV vaccine.

An interesting avenue for exploration is targeting of the *epidermodysplasia verruciformis* (EV)-associated ~30 cutaneous types which have been proposed as a co-factor along with UV radiation in the development of non-melanoma skin cancers (NMSC) [184, 185]. EV is a rare inherited condition that predisposes patients to widespread cutaneous warts and squamous cell carcinoma predominantly caused by HPV5 and HPV8. Both HIV and SOTRs have a higher risk of developing warts, keratotic skin lesions (e.g. actinic keratosis) and NMSC in association with EV type HPV infections [4, 186]. However, the etiologic link to NMSC remains controversial and there are concerns over the timing of infection which appears to occur throughout the lifespan. Recent studies in the mouse system nevertheless are encouraging and vaccination offers an approach to test for an etiologic role for EV type HPV in NMSC in these populations. Taken together, it is now clear that the field of HPV malignancy prevention via vaccination needs to address more types of HPV (i.e. not just HPV16 and 18).

### **Second generation HPV vaccines**

The need for broader protection against cancer-related HPV has spurred the efforts of many laboratories to develop next generation vaccine candidates. Merck & Co, the manufacturers of Gardasil, has created a nonavalent vaccine (V503, targeting the 7 most common oncogenic HPV types in cervical cancer and genital wart types HPV6 and 11) which is now in advanced phase 3 clinical trials (NCT00543543, NCT00943722 and NCT01651949). Preliminary results in a 3-dose vaccine regimen report that the immune responses (with respect to HPV6, 11, 16 and 18) from V503 are non-inferior to Gardasil [187]. Additionally, the nonavalent vaccine prevents approximately 97% of high-grade cervical, vulvar and vaginal diseases caused by 5 additional oncogenic types [188]. However, the cost of manufacturing this

vaccine (and hence its subsequent pricing) is not expected to be cheaper than the current vaccines and this price will likely remain as a limitation towards global implementation.

In light of this, several groups have attempted to simplify the manufacturing process with alternative L1-VLP eukaryotic expression systems such as tobacco plants, yeast or modified insect cells that secrete the VLPs, which together with local low cost manufacturing of a generic product may drive down costs (Reviewed in [189]). Delivery of L1 VLP via live recombinant vectors such as *S. typhi*, measles virus or adenovirus also have great potential because this potentially offers simpler manufacturing, no need for needles/syringes and potentially fewer doses, but enthusiasm is tempered by potential safety concerns with a live vaccine and pre-existing immunity to the vector. In bacterial production systems VLPs are not readily formed; rather the expression of L1 proteins results in VLP sub-units known as pentamers or capsomeres [190, 191]. The neutralizing epitopes of VLP are preserved in capsomeres, and vaccination with capsomeres also induces strong neutralizing antibody responses in animals although lower than for L1-VLP [192]. However, the use of an appropriate adjuvant with capsomeres can achieve neutralizing titers similar to VLPs [193]. Vaccination with L1-expression vectors is another potential approach but efficient *in vivo* delivery and avoiding interference remain significant hurdles.

Given the importance of broad immunity covering all hrHPV, several research groups including ours have investigated the HPV minor capsid protein L2 as a candidate antigen for second generation HPV vaccines. Studies with L2 have shown that the N-terminus contains several highly conserved protective epitopes and is required for several events during the infectious life cycle. Importantly, L2 vaccination induces antibodies that can cross neutralize a large diverse range of HPV genotypes both *in vitro* and *in vivo* [194-197]. Moreover because L2's epitopes are linear, they can be readily expressed in *E. coli*. Unfortunately, co-expression of L1/L2 VLPs results in an L1-response only suggesting L2 is immune-subdominant in the context of a HPV virion [13, 198]. To further complicate matters,

vaccination of L2 alone although broadly neutralizing is still not as immunogenic as the L1-VLPs even with the use of a potent adjuvant. To boost immunogenicity, several groups have attempted several methods including the concatenation of multiple L2 epitopes [123], using scaffolds [199] and alternative display methods of L2 on VLP platforms of papillomavirus [9, 200] or other viruses [201]. While some of these strategies have yielded higher immune responses, the overall responses were still below those of the current VLP, and it is not known if immune titers will be as long lasting as the current vaccine. The apparently weak response to L2 may reflect in part the use of an *in vitro* neutralization assay with poor sensitivity to L2-specific protective antibodies. Nevertheless, it appears that low titers of neutralizing serum antibody are sufficient to confer robust protection in pre-clinical animal models, suggesting that it may not be necessary to achieve similar titers as L1 VLP for effective durable immunity.

#### **Therapeutic HPV vaccines and new immunologic considerations**

Since HPV is a very common sexually transmitted infection, there remains an urgent need for a therapy to effectively treat existing chronic HPV infections and disease. No therapeutic activity has been demonstrated for the licensed HPV vaccines likely reflecting the absence of detectable L1 expression in HPV-transformed tumor cells or basal keratinocytes that harbor the infection. HPV viral oncoproteins E6 and E7 are required for the induction and maintenance of cellular transformation [202], and are consistently and specifically co-expressed in all infected cells including HPV-associated cancers [203, 204]. While the targeting of other viral antigens like E1, E2 and E5 with vaccination is effective for therapy in animal models of disease, it is likely that tumor cells could escape immune responses by loss or down regulation of E1, E2 and E5 expression, but this is not possible for E6 or E7. Therefore, most therapeutic HPV vaccines being tested clinically target HPV E6 and/or E7.

It is clear from natural history studies that most immune competent persons eventually clear HPV infections and low grade intraepithelial lesions, such that the virus becomes

undetectable and the cervical histopathology returns to normal. In contrast, the rate of spontaneous clearance is much lower in immune compromised patients, suggesting that infection elicits a delayed but eventually effective anti-HPV immune response in the majority of patients with an intact immune system [205, 206]. In fact, a subset of high grade intraepithelial lesions does undergo complete regression [205], which is presumably immunologically mediated. While immunotherapies should aim to enhance such anti-viral immunity in those unable to clear HPV naturally, to date, no algorithms exist that can distinguish persons at risk for either high grade dysplasias or invasive disease. Therefore, the standard of care for high grade intraepithelial neoplasia is resection. In a landmark therapeutic clinical trial by Kenter *et al* [207] wherein HPV16+ VIN3 patients were vaccinated with synthetic overlapping long peptides (SLP vaccines) covering HPV-16 E6 and E7, 9 out of 19 patients (47%) exhibited complete regression of the disease and HPV16 specific T-cell responses were detected. Although the regression rate was less than 50%, these findings suggest that it is possible to treat HPV-specific disease and induce complete regression via a vaccine-induced T-cell response.

Unfortunately, monitoring of E6/E7-specific cellular immune responses has proven more complex than for the antibody responses to VLP vaccines ([208-212] and reviewed in [213] and [214]). To date, trials using a plethora of therapeutic vaccine platforms have elicited E6- or E7- specific cellular immune responses in the peripheral blood that were weak and often did not infiltrate the tumor regions. More importantly, even if HPV antigen-specific T-cell responses were detected in the peripheral blood, these responses did not always correlate with clinical response. Recently, Maldonado *et al* [215] suggest that T-cell responses are sequestered in the lesion micro-environment, at the site of antigen. In patients with HPV16+ CIN2/3 primed twice with a DNA vaccine and boosted with a recombinant vaccinia eight weeks prior to a standard therapeutic resection, they found that in subjects that had residual disease, clonally expanded, proliferating effector immune responses that were organized in lymphoid aggregates were localized in lesional mucosa. Intraepithelial CD8+ infiltrates were

increased compared to pre-vaccination, and these infiltrates were associated with histologic features of apoptosis in dysplastic epithelial cells. Vaccinated subjects who had shared HLA alleles had shared T cell receptors (TCRs) in tissue T cells. The frequencies of these TCRs were variable in the peripheral blood, suggesting that the tissue responses were the result of a process of selection, as opposed to transudate. These observations raise the question of whether earlier vaccine studies in which HPV-specific T-cell responses in the blood were very weak or not detectable may have elicited local responses that were not measured. In the heterologous prime-boost vaccination study, within-subject comparisons of tissue samples obtained before and after vaccination suggested that previous studies may have been, in effect, censoring histologic endpoints. Because pre-invasive HPV lesions are clinically indolent, and directly accessible, they present an opportunity to better understand mechanisms of disease clearance, and tissue-localized obstacles to clearance. Based on earlier studies of tissue predictors of clinical outcomes in unvaccinated CIN2/3 lesions that demonstrated down-regulated expression of adhesion molecules in the neovasculature associated with persistent lesions [216], clinical testing of peripheral vaccination with the heterologous prime-boost regimen, in concert with direct manipulation of the lesion microenvironment with topical TLR agonist is ongoing (NCT00788164). Clearly there is much to be learnt about the mechanisms of targeting vaccination responses to the relevant site [217], and that it is beneficial to monitor cellular immune responses systemically and at the site of infection [218].

The negative influence of the local tumor microenvironment on clinical responses to therapeutic vaccination is also becoming more recognized. Much work is currently focused on using different strategies to alter the local microenvironment to enhance immune-surveillance against tumors (Reviewed in [219] and [220]). For example, the local application of the topical immune modulator imiquimod can alter the local microenvironment via activation of innate (TLR7) signaling and foster an effective immune response. Such local inflammatory responses likely enhance targeting to the relevant site and overcome local immune

suppressive responses. Indeed, topical imiquimod treatment is partially effective against genital warts, cervical and vulvar neoplasia [221, 222]. However, as the HPV disease enlarges and progresses, the effects of imiquimod become limited [223]. This situation may require additional and systemic treatments to overcome more profound immune suppression in cancer such as combination therapy of HPV therapeutic vaccination with either chemotherapeutic agents or with low doses of radiation. Indeed, in pre-clinical models, conventional cytotoxic therapies either prime or enhance pre-existing or HPV therapeutic vaccination induced anti-tumor specific immune responses [224-226], but additional work to determine the optimal combinations and timing of administration is needed.

Several factors also limit the action of HPV-specific cytotoxic T cells on infected cells. Immune suppressive cells such as the T-regulatory cells (T-regs) or Tumor-Associated Macrophages (TAMs) [227, 228] are also important contributors immune-suppression locally in the tumor-microenvironment. Studies in cervical cancer also indicate a central role for CD4+ T-regulatory cells in immune evasion [211, 228]. Currently, there is also much interest in using monoclonal antibody-based systemic therapies that target co-inhibitory receptors such as CTLA-4 (Cytotoxic T-lymphocyte-associated protein 4) or PD-1 (Programmed Cell-Death protein 1) on CTLs. Recent studies show the use of anti-CTLA-4 antibodies (e.g ipilimumab or tremelimumab) in clinical trials in several cancers achieved complete clinical responses in some cases associated with the reactivation and infiltration of CTLs into the tumor bed. These findings in turn suggest the potential use of CTLA-4 inhibition in combination with HPV therapeutic vaccination to treat HPV+ cancer.

A second potential target is the recent discovery of PD-1, another inhibitory member of the CD28/CTLA-4 family of co-receptors which is expressed not only in T-cells but other immune cells such as B-cells, macrophages and even NK-cells. Importantly, PD-1 ligands have been shown to be more highly expressed in tonsillar crypts as well as in both HPV-associated head and neck squamous cell cancer (HPV-HNSCC) tumor-associated

macrophages and tumor cells. Concurrently, it was found that the majority of the CD8+ tumor-infiltrating CTLs had high expression of PD-1. Taken together, this provides evidence for the PD-1/PD-L1 pathway in maintaining an immune-suppressive tumor microenvironment and provides a rationale for blocking this PD-1/PD-L1 pathway to improve the immune response against HPV-HNSCC [229, 230]. Surprisingly, however, most cervical cancers are PD-L1 negative [231], implying differences in the immune microenvironment of cervical cancer and HPV-HNSCC.

### **End points**

In HPV vaccine studies, protection against or clearance of high grade CIN (Cervical Intraepithelial Neoplasia stage 2/3) is typically a primary end point since it is the recognized precursor lesion of cervical cancer. However, because of the diagnostic variability for CIN2, many therapeutic studies now focus on CIN3 only to test clinical activity. Therapeutic effects of a vaccine are often delayed because of the time needed to develop an effective immune response as opposed to the more direct action of a small molecule. Therefore it is critical in such studies to follow the endpoints over a sufficient period to capture the effects of vaccination. However, the safety of a delay in treatment of CIN2/3 with LEEP (Loop Electrosurgical Excision Procedure) must be carefully considered because of the risk, albeit low over a limited period, of progression. Indeed, a follow-up of 19 weeks has been safely used, and delays of 9 months for LEEP treatment are taken in routine clinical practice for newly pregnant women with CIN2/3. Care must also be taken when interpreting an effective vaccine response since CIN2/3 patients exhibit significant rates of spontaneous regression (~25%) and inflammation triggered by biopsy may influence responses [205]. An alternative disease upon which to test HPV therapeutic vaccines is VIN2/3 (Vulval Intraepithelial Neoplasia) given its lower regression rate (~1.2%) [232, 233], although this is much less prevalent than CIN2/3. Indeed the low rate of spontaneous regression was used to support the absence of a control group in the landmark HPV16 E6/E7 SLP clinical trial [207].

Similarly, much thought is needed in deciding the end points as well as what constitutes ‘efficacy’ of current prophylactic vaccines. These regulatory and policy-making decisions profoundly affect vaccine development and uptake ([234], reviewed in [235]). The use of CIN2/3 as the endpoint for the qualification of second generation preventive HPV vaccines will greatly increase trial sizes over the use of persistent HPV DNA detection as an endpoint, and may impede their development and drive up cost of bio-similar vaccines. This issue is particularly important for vaccines intended to prevent HPV+ head and neck cancer because there is currently no precursor lesion and screening protocol defined, and it is neither feasible nor ethical to use cancer as an endpoint. However, robust protocols exist for detection of persistent oral hrHPV infection and warrant serious consideration as an endpoint.

The use of immunologic endpoints such as L1 VLP ELISA or *in vitro* neutralization titers in serum might also be considered for non-inferiority studies of biosimilar L1 VLP vaccines, although the correlate of protection has yet to be properly defined. Fortunately there has been minimal, if any evidence of breakthrough infection by vaccine types in appropriately immunized patients. However, this renders the determination of a minimal neutralizing titer associated with protection very difficult, although one possibility is to use the infection by non-vaccine types for which protection is partial and compare serum cross-neutralizing titers in these patients with those who are not infected. It will also be important to understand the role of memory B cells and the recall response in long term protection (i.e. is there sufficient time for the inoculum to elicit a rapid and local antibody response if the local protective antibody level has waned below that required for sterilizing immunity), as this has also been suggested as an important factor. The measurement of relevant immune correlates for therapeutic HPV vaccines is much more controversial especially given the greater technical complexity of the assays, the diversity of effector cells, the importance of targeting of the anti-viral responses to the lesion site and the potential of an immune suppressive local environment. These issues suggest the importance of monitoring the response locally, which



creates significant technical hurdles over measurement of systemic immune responses in blood.

The need for broad protection is becoming increasingly clear in light of differing prevalence of certain key hrHPV in different populations, notably HPV52 and 58, although HPV16 and 18 are the dominant types in cervical cancer worldwide. While there is not clear evidence for competition between types and most mathematical modeling studies suggest genotype replacement is unlikely, one study has recently reported an increase in the prevalence of non-vaccine HPV types after vaccination [236]. As there is insufficient data currently to further substantiate such findings [237], long term follow-ups of vaccinated populations will be required to answer such questions. However, the development of highly multivalent L1 VLP or L2-based second generation HPV vaccines will further reduce concern for such issues.

#### **Screening for HPV-associated malignancies in the era of HPV vaccination**

In considering the impact of HPV vaccination, it is critical to address how it is best integrated with screening. Vaccination with L1 VLP will not render cervical screening redundant in the near term as it has no therapeutic effect. In addition, screening is not recommended for women >26 years (based on the assumption that they have had an active sexual history), and vaccination is recommended for 9-26 year olds, i.e., many older women have not benefitted from HPV vaccination. Furthermore, because of the limited hrHPV type specificity of the licensed vaccines, screening will still detect disease associated with non-vaccine types. However, these screening programs must be re-evaluated since the predictive value and cost-effectiveness of screening will be significantly lower in vaccinated women [238] (reviewed in [239]), and dramatically so with the advent of the nonavalent vaccine.

To begin to address such issues, it has been proposed that primary screening be done via HPV DNA testing first followed by Pap cytology triage (Reviewed in [240], [241]). These HPV DNA detection assays are more sensitive for disease as compared to PAP screening; however,

in terms of determining true disease, the assays are at least 10% less specific than the Pap smear [242]. Nonetheless, cohort studies have subsequently shown that there is actually minimal over-diagnosis when HPV DNA testing is used as the sole modality with cytology reserved for triage of HPV-positive women with increased screening intervals (e.g. once every 3 years) [243, 244]. Indeed, the US FDA has recently approved the Roche cobas HPV test methodology as the first HPV DNA test for primary cervical cancer screening (there are current 4 FDA-approved assays but only 1 is approved for primary screening). The cobas® test specifically identifies HPV 16 and HPV 18, while concurrently detecting 12 other types of high-risk HPVs. It will be up to professional medical societies and organizations to determine how this HPV DNA test will be incorporated into current screening protocols. Logically, DNA testing combined with cytology will probably be the best regimen for developed countries with robust healthcare infrastructure, and might be used to trigger treatment with a therapeutic HPV vaccine. Many resource-limited developing countries have adopted visual inspection by acetic acid (VIA)-only programs as it is a cheaper and immediate point-of-care approach compared to delayed HPV DNA/Pap testing in a central laboratory, although clearly less predictive and potentially problematic to combine with an immunotherapy without knowledge of the hrHPV genotype (assuming a type-specific immunotherapy).

In contrast to the cervix, measures to detect and screen for HPV-associated oropharynx cancers have been lacking due to a lack of well-defined precursor lesion (reviewed in [245]). While primary prevention via vaccination should prove promising in the long term [246], there is currently no secondary prevention option. Recently, a strong association between HPV16 E6 serum antibodies and HPV-associated oropharynx cancers was reported [247]. Furthermore, these HPV16 E6 antibodies were present in a notable proportion of patients with HPV-associated oropharyngeal cancers for >10 years before diagnosis, but this approach currently lacks sufficient predictive value alone. HPV DNA testing in oral cavity specimens [248] is a particularly promising approach for screening, although more work is needed to understand the predictive value of this bio-marker testing strategy for HPV+ HNSCC and its

precursors. HPV malignancies of the oropharynx are predominantly due to a single HPV type , HPV16, and thus an immunotherapeutic approach focused on HPV16 T-cell based vaccines could be the primary goal for immunotherapeutic control of these infections, particularly in those were have seroconverted to HPV16 E6 positivity.

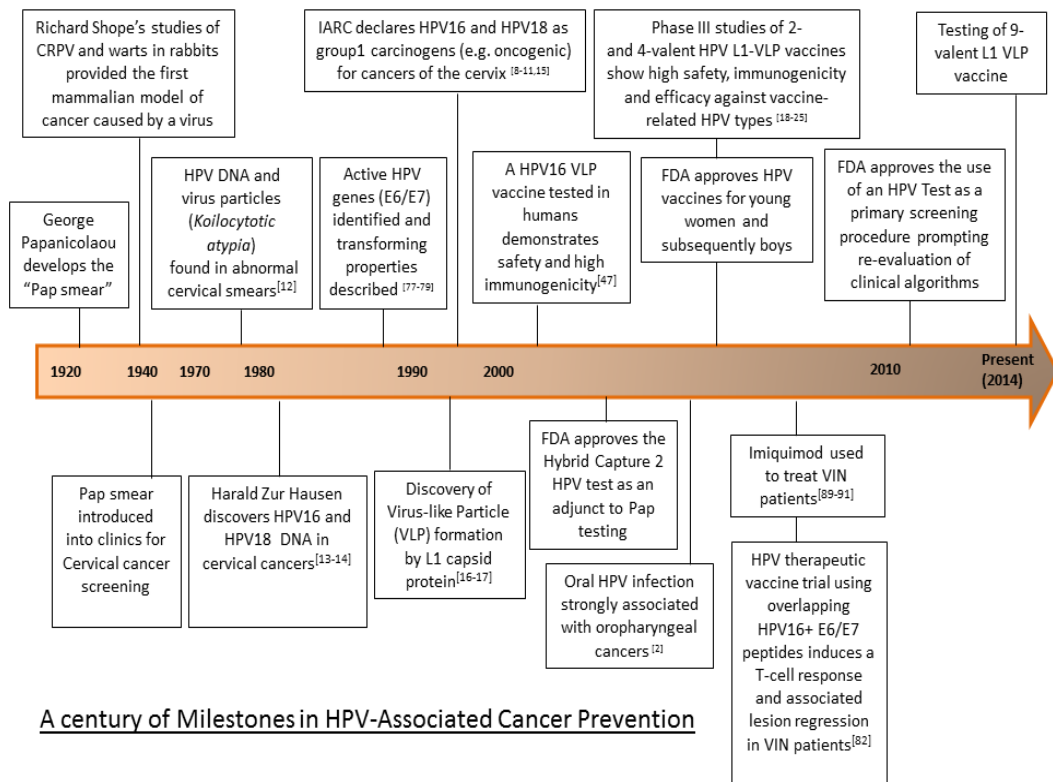


Figure 6. A summary of milestones over a century of human papillomavirus research and implementation of measures to prevent cervical cancer.

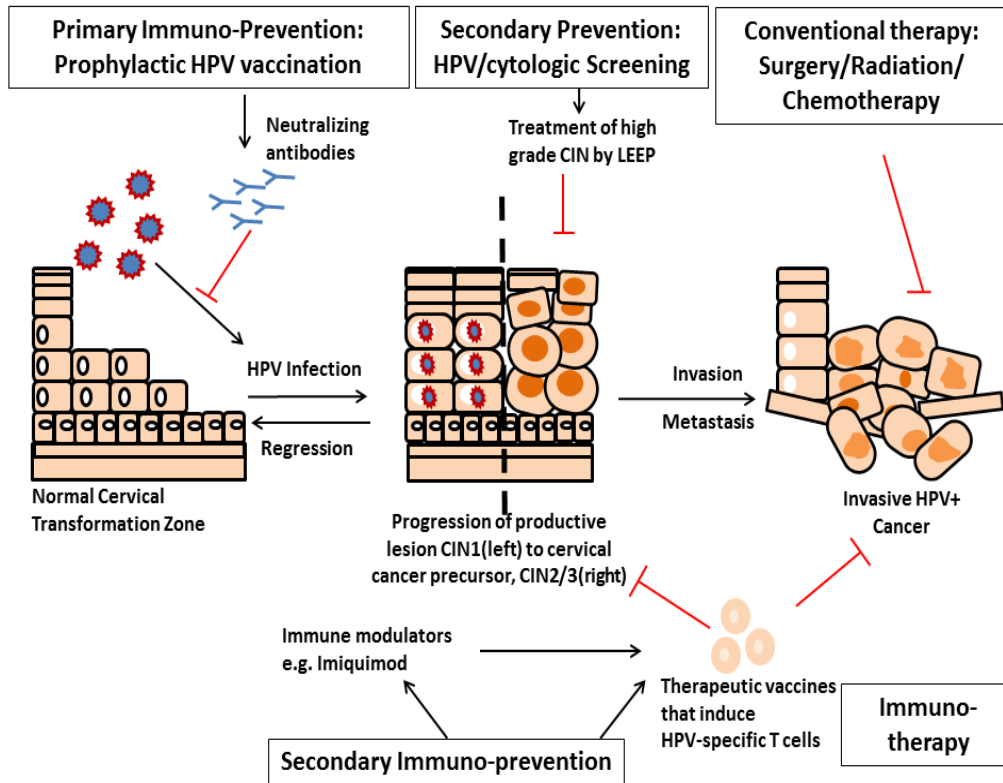


Figure 7. A diagram summarizing the stepwise progression from HPV infection of the cervical transformation zone to cervical cancer and the opportunities for intervention.

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## **B. Chapters**

### **I. Preparation and properties of a papillomavirus infectious intermediate and its utility for neutralization studies**

#### **ABSTRACT**

We show that minor capsid protein L2 is full length in clinical virion isolates and prepare furin-cleaved pseudovirus (fcPsV) as a model of the infectious intermediate for multiple human papillomavirus (HPV) types. These fcPsV do not require furin for *in vitro* infection, and are fully infectious *in vivo*. Both the  $\gamma$ -secretase inhibitor XXI and carrageenan block fcPsV infection *in vitro* and *in vivo* implying that they act after furin-cleavage of L2. Despite their enhanced exposure of L2 epitopes, vaccination with fcPsV particles fails to induce L2 antibody, although L1-specific responses are similar to PsV with intact L2. FcPsV can be applied in a simple, high-throughput neutralization assay that detects L2-specific neutralizing antibodies with >10-fold enhanced sensitivity compared with the PsV-based assay. The PsV and fcPsV-based assays exhibit similar sensitivity for type-specific antibodies elicited by L1 virus-like particles (VLP), but the latter improves detection of L1-specific cross-type neutralizing antibodies.

#### **INTRODUCTION**

Human Papillomavirus (HPV) is one of the most common sexually transmitted infections, and persistent infection with ~15 'high risk' HPV genotypes (most often HPV16, HPV18, HPV31, HPV45) frequently can cause high-grade intraepithelial neoplasia. Left untreated, these high-grade lesions can progress to invasive carcinoma of the cervix and other anogenital regions and oropharynx. Indeed, HPV is the etiologic agent responsible for 5% of all cancer deaths worldwide, including 99% of cervical cancers [1, 249]. As the two prophylactic HPV vaccines are licensed for protection against only two oncogenic HPV types (HPV16 and HPV18), the development of new inhibitors and second generation HPV vaccines has continued.

The study of papillomavirus has been technically difficult because completion of the PV life cycle requires squamous differentiation of the infected keratinocyte that is not replicated by standard tissue culture conditions. However, organotypic raft culture causes infected keratinocytes to undergo squamous differentiation and thus generates infectious PV [16, 250, 251]. This method produces limited quantities of virions containing the authentic viral genome for which there is no simple per cell infectivity assay. An alternative approach is the production of PV pseudovirion (PsV) by the co-transfection of the 293TT cell line with codon-modified L1 and L2 expression vectors and a reporter plasmid genome [252]. The cells are lysed 48 hours later, and incubated overnight at 37°C (known as the maturation step) before being purified by density gradient ultra-centrifugation. These purified PsV can be readily used for surrogate infectious studies both *in vitro* and *in vivo* because they deliver a reporter construct, typically expressing luciferase or GFP, or alternatively the PV genomes can be encapsidated in this system to produce quasivirions (QV) [17, 18, 253-255].

Residues 17-36 of minor capsid protein L2 are buried below the capsid surface of HPV16 PsV, inaccessible to the neutralizing monoclonal antibody RG1 [68], but become accessible to RG1 as early as four hours in the infectious process [45]. For exposure of the RG1 epitope, PV must first undergo a conformational change and adopt an intermediate structure. This is triggered by binding of virions to heparan sulphate proteoglycans (HSPG) on the basement membrane (that has been revealed upon wounding the epithelium) and cleavage of amino acids 1-9 at the N-terminus of L2 by furin. This conformational change in the capsid is also modeled *in vitro* by the association of PsV with extracellular matrix (ECM) produced by certain cell lines, e.g. HaCaT and MCF7, although not 293TT cells to which the PsV bind directly via HSPGs [45, 65]. Importantly this difference in mechanism of L2 exposure upon binding of PsV to 293TT cells has been linked to poor sensitivity in L2-, but not L1 VLP-specific antibody-dependent *in vitro* neutralization assays using this cell line [11, 44]. Indeed, the discord between the low or undetectable neutralization titers measured using this system



despite robust ELISA reactivity and protection upon passive transfer and PsV challenge of mice with the same L2-vaccinated sera, suggest the need for improved assays that use target cells other than 293TT to better replicate the uncloaking of L2 observed during infection *in vivo*.

Studies of the PsV production procedure show that HPV PsV particles which do not undergo the maturation step are more susceptible to neutralization by L2 antibodies, suggesting L2 is initially exposed during the early events of packaging and co-assembly with L1 capsomeres but is slowly “buried” in the capsid structure as the virus matures into a more stabilized form [22, 256]. In contrast, studies of organotypic raft culture-derived virions show more mature HPV virus particles (i.e. virions from a 20-day old raft) harvested from cornified layer, are more susceptible to neutralization by L2-specific antibodies compared to virions harvested from the suprabasal layer of the tissue rafts cultured for 10 days [257]. These findings suggest differential exposure of L2 epitopes on the capsid during virion morphogenesis and elevation from the more reducing environment of the suprabasal layers to the upper oxidizing cornified layers in the differentiated tissue raft culture [16, 257]. Whereas some studies analyzing L2 in virions purified from warts suggest L2 is full-length, others show L2 existing in a doublet by immunoblot [25, 28, 29]. While the latter may reflect partial degradation during virion purification, it remains possible that a subset of wart-derived infectious virions exhibit variable degrees of L2 exposure and furin-cleavage.

There are important implications if a subset of L2 is already exposed and cleaved by furin even before transmission and encountering the host. Firstly, partially pre-cleaved HPV can infect both HSPG- or furin-deficient cell lines [45, 258]. This shows that changes in the conformation of the capsid associated with L2 exposure and cleavage allows HPV to become independent from cellular factors considered to be required for infection. Importantly, carrageenan (a type of sulfated polysaccharide extract from red algae which is used in sexual lubricants) as well as inhibitors of  $\gamma$ -secretase (e.g. XXI) [91, 259] were recently identified as

potent inhibitors of mucosal trophic HPV types [260-262]. However, it is currently unknown if their potency would be compromised if a subset of, or the true infectious form of native virions is in the L2-cleaved conformation. A second implication is that potential differences in L2 cleavage within organotypic raft-derived virion and PsV preparations may account for reported differences in sensitivity to inhibitors and the impact of mutations in the RG1 epitope [38, 263].

Here we describe a method to generate milligram quantities of highly (~90% of L2) furin-cleaved pseudovirus (fcPsV), and examine its immunogenicity and the impact of carrageenan and furin and  $\gamma$ -secretase inhibitors upon infectivity of the furin-cleaved intermediate. To confirm if furin cleavage of L2 represents an infectious intermediate, we examined the cleavage status of L2 in several wart-derived virions of divergent PV genotypes. Further, we assessed if use of fcPsV for *in vitro* neutralization studies could enhance the sensitivity for L2-specific neutralizing antibodies in a high throughput format without compromising measurement of L1 VLP-specific antibody.

## **MATERIALS AND METHODS**

### **Ethics Statement**

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies were performed with the prior approval of the Animal Care and Use Committee of Johns Hopkins University (protocol MO08M19). Human tissue samples were collected following informed consent of the patient or the patient's guardian in accordance to the Ethics Committee of the Medical University Vienna (ECS 1327/2012).

### **Plasmids**

The plasmid vectors pShell expressing codon optimized L1 and L2 capsid genes of HPV16, 45 and 58 were kind gifts from John Schiller, NCI. Additional PsV genotypes HPV6, 11, 18,

31 and 33 codon optimized L1 and L2 capsid genes were sub-cloned into double expression vector pVITRO1-neo-mcs (Invivogen, San Diego CA). The human furin cDNA (NM\_002569.2) was obtained from Sino Biological Inc and was sub-cloned into pIRESpuro2 (Clontech Laboratories Inc, USA) between the *Afl*III and *Bst*B1 restriction sites after PCR amplification using primers 5'-GAGAGACTTAAGATGGAGCTGAGGCCCTGG (Forward) and 5'-GACCGATTCGAATCATCAGAGGGCGCTCTGGTC (Reverse) to yield Furin-pIRESpuro2. Mouse papillomavirus (MmuPV1) plasmids pMusPV, pMuL2w, pMusSheLL and the SV40 T-antigen plasmid, pTIH were kind gifts from Christopher Buck, NCI. MmuPV1-L2 was sub-cloned into pet28a(+) vector (Novagen, San Diego, CA) between the BamH1 and Xho1 restriction sites after PCR amplification of codon optimized MmuPV1-L2 gene from pMuL2w using primers 5'-GAGAGAGGATCCATGGTCAGCGCCGATCGCTCA (Forward) and 5'-GACCGACTCGAGGTA GATCCTGTACTTCCGCTTGCGCTT to yield MmuPV1-L2-pet28a(+).

#### **Cell culture and creation of cell lines 293TTF and LoVoT**

All cell lines were maintained in DMEM supplemented with 10% FBS, 1X penicillin and streptomycin, 1X Non-essential amino acids and 1X Sodium Pyruvate (Gibco Life technologies, Grand Island NY). For the creation of 293TTF and LoVoT,  $5 \times 10^6$  293TT or LoVo cells/well respectively were seeded into a 6 well plate the day before transfection. Individual wells were transfected with either no template (mock transfection) or either Furin-pIRESpuro2 into the 293TT cells or pTIH into the LoVo cells respectively using Mirus TransIT 2020 (Mirus Bio, Madison WI) according to the manufacturer's protocol. After 48 hours, the cells were harvested using 0.5% trypsin and each well re-seeded into individual 10cm<sup>2</sup> plates. Both control and transfected cells were treated with complete growth media with either puromycin concentration of 2µg/ml for 293TT cells, or with 200µg/ml Hygromycin B for LoVo cells. Fresh media was introduced every 4 days until all the control cells died and the appearance of clonal colonies in the transfected plate. Colonies were picked

using cloning cylinders (Milipore) as per manufacturer's recommendations and expanded. The furin-deficient cell line FD11 [264] and the FD11-F cell line in which furin expression is complemented by ectopic expression of the furin gene in FD11 cells, were both gifts from Tae Heung Kang and TC Wu (Johns Hopkins University, Bethesda, MD). The LoVo cell line [265] was a kind gift from Weijie Poh and James Herman Lab (Johns Hopkins University, Bethesda, MD). PGSA-745 cells [266] were a kind gift from John Schiller (National Cancer Institute, Bethesda, MD).

### **Pseudovirion (PsV) and Furin-cleaved Pseudovirion (fcPsV) Production**

Standard PsV were generated in 293TT cells following the previously described production protocol (<http://home.ccr.cancer.gov/Lco/pseudovirusproduction.htm>). Firefly luciferase expression plasmid was employed as the reporter for PsV infection in both neutralization assays and for mice vaginal challenge studies. Furin-cleaved PsV (fcPsV) were generated by following the same standard PsV production protocol but with the following modifications: (1) 293TTF cells were used instead of 293TT cells; (2) Maturation buffer did not contain ammonium sulfate; (3) CaCl<sub>2</sub> was added to the maturation buffer to 5mM; (4) Maturation was carried out for 48 hours instead of 24 hours. The protocol was also suitable for producing fcPsV encapsidating GFP or SEAP reporter and the former was used in time-course assays. Transmission electron microscopy (TEM) analysis on the purified virions were performed as described in [30]

### **Generation of infectious mouse papilloma virions (MmuPV1) and mouse papilloma tail lesions**

The synthetic MmuPV1 genome plasmid- pMusPV was excised from its vector backbone and religated by adapting a previously described papillomavirus re-ligation protocol (<http://home.ccr.cancer.gov/lco/religation.htm>). Co-transfection of pMusSheLL containing the codon optimized sequences of MmuPV1 L1, L2 and the re-ligated MmuPV1 genome into 293TT cells and following remaining steps of the standard PsV protocol to produce infectious

MmuPV1 virions. Five athymic nude mice (NCr-nu/nu) were subsequently challenged on their tails with 200 $\mu$ L of the purified MmuPV1 virion as described in [267]. Papilloma-like lesions were observed on the tails subsequently after 5 weeks. These mice were then euthanized and mouse papillomas were collected, snap frozen in liquid nitrogen and stored at -80°C until further use.

### **Western blot analysis and quantification**

Primary antibodies used for L2 detection were mouse monoclonal antibody RG1 [68] to HPV16 L2 17-36, or rat monoclonal antibody WW1 that recognizes the same epitope and also neutralizes HPV16 (Wu *et al.*, 28th International Papillomavirus Meeting, Puerto Rico, Nov 30, 2012, Abstract B06-086). Primary antibody for furin and T-antigen detection was respectively B-6 and PAB101 (sc-133142 and sc-147, Santa Cruz Biotechnology, Dallas, TX). Respective secondary antibodies include HRP-goat anti-mouse IgG light chain or HRP-donkey anti-rat IgG Heavy+Light chain (Jackson ImmunoResearch, West Grove PA). For 293TT and 293TTF supernatant analysis, cells were seeded in DMEM (10% FBS, 1X penicillin/streptomycin) at 500,000 cells/well in a 6 well plate in the presence or absence of puromycin (2 $\mu$ g/ml). After 48 hours, the wells were washed 3 times with PBS and serum free media (plus puromycin 2 $\mu$ g/ml for 293TTF) was added for 24 hours. Subsequently, media was collected and spun at 1600 rpm for 4.5 minutes at room temperature to remove any debris. The supernatant was concentrated using 50kDa Centricon concentrators (Milipore, Billerica, MA) and subsequently used for western blot analysis. Intensity of bands was analyzed using the NCI software ImageJ (<http://rsb.info.nih.gov/ij/index.html>). For comparison of L2 bands in the various clinical warts samples, virions were extracted from the samples using the following protocol (<http://home.ccr.cancer.gov/lco/VirionExtraction.htm>). HPV26, HPV57 L2 was detected using WW1, HPV6 L2 was detected using L2 $\alpha$ (11-88)x8 rabbit serum made as described in [8], BPV-1 L2 was detected with mouse monoclonal antibody C6, and MmuPV1 L2 was detected with mouse anti-MmuPV1-L2 sera.

### **L1/L2 VLP vaccination and generation of MmuPV1-L2 anti-sera**

Groups (n=10) of 8-10 week old female Balb/c mice were vaccinated s.c. three times at two week intervals with L1/L2 VLP (5µg), or furin-cleaved L1/L2 VLP (5µg) formulated with alum (50µg) and MPL (5µg), or 50µL of Cervarix™, or PBS alone, or PBS with adjuvants alum (50µg) and MPL(5µg). Blood samples were collected two weeks after the second and third vaccination. For MmuPV1-L2 immunization, MmuPV1-L2 protein was synthesized by bacterial induction using the MmuPV1-L2-pet28a(+). Following protein induction and purification and dialysis (See [7] for full method details), five 8-10 week old female Balb/c mice were vaccinated s.c with 25µg of purified MmuPV1-L2 protein formulated with alum (50µg) and MPL (5µg) three times at two week intervals. Blood was collected a week after the first boost. In both immunization regimens, following blood collection, the blood samples were clotted o/n at 4°C and serum was collected after centrifugation at 2,000g for 10min at 4°C.

### **ELISA**

For analysis of antibody response against HPV16 L1-VLP and L2 full length protein, maxisorp microtiter 96-well plates (Thermo Scientific Nunc, Waltham MA) were coated with either L1-VLP or L2 protein at 500ng in 100µl PBS/well and incubated overnight at 4°C. The next day, plates were blocked with PBS/1% BSA for 1 hour at 37°C. Serum samples diluted 1:50 in PBS/1% BSA were then added to the plates for 1 hour at 37°C. Following this, plates underwent 3 washes with washing buffer (0.01% Tween 20 in PBS) before HRP-sheep anti-mouse IgG diluted 1:5000 in 1% BSA was added to each well and plates were incubated for 1 hour at 37°C. After 3 further washes, 100ul of ABTS solution, 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid] (Roche, Basel Switzerland) was added to each well for development, and absorbance at 405nm read using a Benchmark Plus (Bio Rad, Hercules CA).

### ***In vitro* Neutralization Assays**

Serum samples (4 $\mu$ L) were serially diluted two-fold in culture media, and mixed with HPV PsV or fcPsV (0.1 $\mu$ g/ $\mu$ L of L1) carrying luciferase reporter plasmid. Mixtures were incubated at 37°C for two hours, added to 293TT, FD11 or LoVoT cells that had been plated at 15,000 cells/well one day prior. Approximately, 5-fold more fcPsV was required for assays using LoVoT and 50-fold for FD11 cells as compared to 293TT cells e.g. at a 1:5000 or 1:1000 dilution of virus for 293TT and LoVoT respectively. These plates were incubated at 37°C. After 72 hours, cells were lysed with 30 $\mu$ L of Cell Culture Lysis Reagent (Promega, Madison WI) for 15 min at room temperature on a rocking platform. The entire lysates were transferred to a 96-well black plate, and luciferase activity was measured by adding 50 $\mu$ l of luciferin substrate to each well (GloMax<sup>®</sup>-Multi Detection System, Promega, Madison WI). The same procedure was carried out for FD11 or LoVoT based neutralization assays.

### **Pull down assays**

Immuno-precipitation studies were performed using Dynabeads<sup>®</sup> Protein G (Life Technologies, Grand Island NY) according to the manufacturer's protocol with some adjustments. Briefly, 10 $\mu$ g of RG-1.1 monoclonal antibody was mixed with 1.5mg of Dynabeads protein G and incubated on a rotator for 30 minutes at room temperature. Subsequently, equal amounts of purified fcPsV and PsV based on L1 content were added and the mixture incubated on a rotator overnight at 4°C. Washing and elution of bound virions were performed as per the manufacturer's protocol. Elutes were subjected to western blot analysis.

### **Neutralization and Inhibition assays**

293TT, LoVoT or PGSA-745 cells were seeded at 15,000 cells/well in 100 $\mu$ L of medium in a 96-well plate and incubated overnight. The next day, for neutralization assays, using another 96-well plate, the serum to be tested was serially 2-fold titrated across the plate in a total volume of 50  $\mu$ L. Following this, 50 $\mu$ L of equivalent amounts of PsV and fcPsV based on L1

amount was added to make the antibody-virus mixture 100  $\mu$ L. The plates were incubated at 37°C for 2 hours before the mixture was added onto the pre-plated cells and incubated for 72 hours. The total volume of 200  $\mu$ L resulted in the starting dilution for most L2 serum was at 1:50 while L1-serum was at 1:200. For inhibitor assays, using another 96-well plate, the relevant inhibitors in either sterile water or DMSO were subjected to a 2-fold serial dilution with the highest dilution starting at 256 $\mu$ g/ml. Titrated PsV in 50 $\mu$ L was then added to the diluted candidate inhibitor and this mixture (100  $\mu$ L) was immediately added to the pre-plated cells. Regardless of assay, the plates were incubated for 72 hours and cells were lysed with 30  $\mu$ L of Cell Culture Lysis Reagent (Promega, Madison WI) for 15 min at room temperature on a rocking platform. The entire lysates were transferred to a 96-well black plate, and luciferase activity was measured by GloMax<sup>®</sup>-Multi Detection System (Promega, Madison WI) after adding 50  $\mu$ L of luciferin substrate (Promega, Madison WI) to each well. IC50 values or titers were calculated using Prism (GraphPad Software, San Diego CA). Approximately, 5-fold more fcPsV was required for assays using LoVoT and PGSA-745 cells as compared to 293TT cells.

For the time course infectivity assay, 293TT cells were pre-plated at 60,000 cells/well in a 24 well plate and incubated at 37°C overnight. The next day, equal amounts of HPV16 PsV or fcPsV16 (based on L1 amount) encapsidating a GFP reporter plasmid was added to the cells. Following this, at time points 24,48 and 72 hours after addition of virus to the cells, the cells were harvested with trypsin-EDTA (1x) (Gibco, Life technologies, Grand Island NY) , and washed with 1ml of fluorescence-activated cell sorter (FACS) buffer (0.5% BSA in PBS, pH 7.4). The cell pellet was then resuspended in 300 $\mu$ L of 1xFACS buffer and GFP expression as an indicator of infectivity was analyzed by flow cytometry with a Becton Dickinson FACSCalibur. The experiment was performed in triplicate and % infectivity data was analyzed using CellQuest software (Becton Dickinson Immunocytometry).



### **Mouse vaginal challenge studies**

Four days before vaginal challenge, 8-10 weeks old Balb/c mice purchased from NCI were subcutaneously injected with 3mg of medroxyprogesterone (Depo-Provera, Pfizer, New York NY). PsV or fcPsV stock amounts were standardized based on their L1 content. Each mouse was challenged with 2µg of PsV or fcPsV (based on stock virus with L1 content of 0.2µg/µl) which was pre-mixed for an hour with the relevant inhibitors or an equal volume of diluents with 3% carboxymethyl cellulose (CMC) except for the 1% carageenan for which CMC was omitted. The amount of Heparin used (based on previous data) was 1000-fold in excess to the L1 content [65]. Approximately, 200µM of Gamma secretase inhibitor XXI was used premixed for one hour with either PsV or fcPsV that were instilled into the vaginal vault before and after cytobrush treatment (15-20 rotations, alternating directions) while the mice were under isoflurane anesthesia. Forty eight hours after challenge, mice were anesthetized by isoflurane, and 20µL of luciferin substrate (7.8mg/ml, Promega, Madison WI) was delivered into the vaginal vault before imaging. Bioluminescence was acquired for 10 min with a Xenogen IVIS 100 (Caliper Life Sciences, Hopkinton MA) imager, and analysis was accomplished with Living Image 2.0 software. For  $\gamma$ -secretase inhibitor studies, imaging was performed 72 hours post-challenge.

### **Statistical Analysis of Neutralization assay titers**

For comparison of 293TT assay against the FD11 assay (Table 1), individual mouse sera from (30) vaccinated with the respective L2 vaccine candidate titers were analyzed. Neutralization titers (the reciprocal of the dilution that causes 50% reduction in luciferase activity) were recorded for each individual serum and the sample average was calculated within each group. Nonparametric Wilcoxon signed-rank test was performed to detect statistical significance between the two groups. For comparison of 293TT assay against LoVoT assay (Table 2), the same mouse serum samples from [8] were pooled and underwent inter-assay triplicate comparison. Neutralization titers (EC50) reported are the mean of this triplicate testing. The non-linear model  $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC50} - X) * \text{HillSlope}))}$  was fitted to

log-transformed neutralization titers data. The estimated EC50s and their 95% confidence intervals are reported. Statistical calculations were performed using GraphPad Prism version 6.

## RESULTS

### **Generation of 293TTF, a clonal cell line that overexpresses enzymatically active furin**

L2 in HPV16 PsV could be cleaved if furin is added during the maturation step of the standard HPV PsV protocol. However, the extent of L2 cleavage in the virions was only approximately 35% in these preparations[258]. This prompted us to develop an alternative approach to reproducibly produce fully furin-cleaved PsV at high titer. To this end, a clonal 293TT cell line that over-expresses furin, termed 293TTF, was generated from 293TT cells that is used for conventional PsV production (Figure 8).

Furin is present in 293TT cells but the amounts were below the limit of detection (Figure 8A, lane 1). This was consistent with the literature on furin being highly regulated and not readily detected via western blot methods [268, 269]. However, Western blot analysis of 293TTF cells produced a prominent band of 90-100kDa, a size consistent with an unresolved doublet of the endogenous immature/pro-furin (96kDa) and mature furin (90kDa) (Figure 8A, lanes 3, 4). Image analysis by densitometry showed that the total amount of furin expressed in 293TTF was at least 150-fold higher than in parental 293TT cells. Another band was observed at ~60kDa which was reported in the antibody material data sheet as a furin splice variant (Figure 8A, lanes 1, 3, 4). The level of secreted furin released by 293TTF cells was also 200-fold higher compared to 293TT cells (Figure 8A, lane 5-7). Secreted furin has a lower molecular weight (~80kDa) than cell-associated furin due to cleavage of its C-terminal transmembrane region.

### **Production and analysis of HPV furin-cleaved pseudovirus (fcPsV)**

To assess if 293TTF can act as a producer cell line for furin-cleaved pseudovirus (fcPsV), we performed the standard PsV production protocol using either 293TT or 293TTF. Particles purified from each preparation using Optiprep™ step gradients were morphologically indistinguishable when stained with uranyl acetate and viewed by transmission electron microscopy (Figure 9A-B).

To examine HPV16 fcPsV functionally, we tested whether fcPsV produced from 293TTF could bypass the requirement for furin and thus infect furin-deficient cell lines such as FD11 (Chinese hamster ovary-CHO cells with furin gene knocked out)[264] and LoVo (a human colon adenocarcinoma line)[265]. In FD11 cells, the infectivity of HPV16 fcPsV was 2 logs higher than HPV16 PsV (Figure 8B). Importantly, the infectivity of HPV16 PsV was restored to levels similar to that of fcPsV in FD11-F cells which are FD11 cells re-complemented for the wild type furin gene (Figure 8C). A similar trend was observed with LoVo cells versus LoVo supplemented with purified exogenous furin (data not shown). HPV16 fcPsV and PsV infectivity in 293TT was also tested with and without the presence of a furin inhibitor (20µM). Both HPV16 fcPsV and PsV were similarly infectious in 293TT cells (Figure 8D), whereas upon furin inhibition, HPV16 PsV infection decreased dramatically by 2 logs. Interestingly, HPV16 fcPsV infection was also affected whereby the infectivity decreased almost 1 log-fold compared to fcPsV infection without inhibitor. This observation suggests that not all the virion particles in the 293TTF-made fraction were cleaved (Figure 8E).

We next performed a time course infectivity experiment with equal infectious units of GFP-encapsidated HPV16 PsV and fcPsV to assess if furin-cleaved viruses delivered the marker gene more rapidly since furin cleavage has been shown to be a rate-limiting step for PV infectivity. The infectivity of HPV16 fcPsV is higher at 24 and 48 hours, there was no significant difference from the PsV at 72 hours (Figure 9C), consistent with the wave of PsV infection catching up with the fcPsV. Similarly, the difference in infection of luciferase-

encapsidated HPV16 fcPsV and PsV after 72 hours *in vivo* when tested in a murine vaginal challenge model was not significant (Figure 9D). These findings are consistent with fcPsV as an infectious intermediate, and suggest that the altered purification protocol does not damage the structure or infectious potential of fcPsV.

The furin inhibition results observed with HPV16 fcPsV in figure 8E prompted us to check the extent of L2 cleavage in HPV16 fcPsV made with 293TTF using the standard HPV PsV protocol. Densitometry results showed approximately half of the L2 of HPV16 fcPsV was cleaved after 24 hours maturation in these preparations from 293TTF cells, whereas cleavage of L2 in PsV prepared in 293TT was not observed (Figure 14A). The partial cleavage of L2 supports the notion that not all virion particles are cleaved hence explaining why there is a small but detectable inhibition by furin seen in figure 1E. More importantly, it was surprising to observe only partial cleavage of L2 despite 150-fold more furin in 293TTF (Figure 8A). We rationalized this may reflect inappropriate conditions for furin proteolytic activity.

Indeed, studies on furin enzymology found that *in vitro* processing and activation of transfected furin in cell lysates was optimal at pH6.5-7 with a calcium concentration of 5mM [269-271]. To optimize the maturation conditions to obtain more fully cleaved L2 in fcPsV, we tested a range of calcium chloride concentrations (2.5, 5 or 7.5mM) with 24 hours maturation (Figure 9E). Our results indicated that addition of CaCl<sub>2</sub> beyond 5.0mM calcium concentration was not beneficial. This was further substantiated in an infectivity test using 293T cells whereby it was shown that the infectivity of fcPsV virions matured with 5mM CaCl<sub>2</sub> was almost the same as its no furin-inhibitor counterpart. In contrast, the infectivity results for HPV16 fcPsV matured without 5mM CaCl<sub>2</sub> in the presence or absence of furin inhibitor was similar to the results seen in Figure 8E and Figure 14. Together, this suggests the addition of calcium improves furin cleavage of L2 which in turn significantly increases the proportion of fcPsV. To further ensure that cleavage of L2 occurs to the greatest extent possible, we also extended the maturation time to 48 hours.. Subsequently, we observed that

the L2 cleavage of HPV16, HPV18, HPV45 and HPV58 fcPsV generated using 48h maturation with 5mM CaCl<sub>2</sub> supplementation was at least 80%, whereas L2 remained intact in PsV prepared using the standard protocol (Figure 9F). Likewise, the fcPsV of all four types were able to infect furin-deficient cells, whereas conventional PsV infected FD11 cells minimally (data not shown).

### **L2 is full length in natural papillomavirus virion isolates**

Whereas some studies analyzing L2 in virions purified from warts suggest that it is full-length, others show L2 existing in multiple forms by Western blot analysis [25, 28, 29]. These lower molecular weight forms were explained as partial but unnatural degradation that occurred during the virion purification process. However, it is possible that a subset of wart-derived infectious virions contain furin cleavage that represents the fully mature form of the virus. Thus, to discern if furin-cleaved PV truly represents an intermediate conformation, we compared the molecular weight of L2 in regular PsV which are known to be uncleaved with L2 in PV derived from human and animal clinical wart samples. Side-by-side comparisons showed that PV L2 is uncleaved as only a single band of the same size was observed (Figure 3). In some preparations, significantly lower molecular weight L2 bands were also detected near the 50-55kda region (Figure 10C-D). However, based on our purified fcPsV experiments which distinguishes furin-cleaved and uncleaved L2, these lower L2 bands are not the furin-cleaved form of L2, but likely rather L2 degraded during the preparation. Thus the furin-cleaved form is an intermediate produced during the infectious process rather than the fully mature infectious form. Taken together, the results thus far indicate that fcPsV manufactured in 293TTF cells are consistent with an intermediate conformation and potentially useful to study its biology.

### **Impact of carrageenan and heparin on fcPsV infectivity**

It was previously suggested that both heparin and carrageenan are highly inhibitory to HPV infection via blocking binding to and conformational changes in the virion on the ECM [18,

261]. Since furin cleavage occurs after PV adopts in its intermediate structure, the use of fcPsV particles would allow us to assess whether these inhibitors act downstream of these events. Indeed, we hypothesized that neither carrageenan nor heparin would be inhibitory for HPV fcPsV infection as there is no further need of fcPsV to undergo primary binding to the extracellular matrix, as required to facilitate the conformational change and L2 cleavage events that fcPsV have already undergone. Unexpectedly, infection by fcPsV was still potently inhibited by both heparin and carrageenan in 293TT cells with similar IC<sub>50</sub> values to PsV which were used as a control (Figure 11A-B). These IC<sub>50</sub> values were also consistent with published literature using HPV PsV [65, 261] and the same degree/pattern of inhibition of infection was observed in the mouse vaginal challenge model using inhibitor concentrations that were previously published (Figure 11D) [65, 261].

Both heparin and carrageenan could also inhibit fcPsV16 infection of PGSA-745 cells at high concentrations (Figure 11C). Interestingly, at lower concentrations of heparin or carrageenan, the infectivity of fcPsV16 was enhanced slightly in heparin-deficient PGSA 745 cells (Figure 11C) but not in 293TT cells (Figures 11A-B). This surprising phenomenon suggests that high quantities of either heparin or carrageenan completely coat the virion and block all receptor binding. Conversely, partial coating of fcPsV with heparin or carrageenan may either enhance secondary L1 conformational changes or bridge the interaction with HSPG-deficient cells but not cells with normal levels of HSPG. This observation is also consistent with recent evidence [64, 272, 273] showing that soluble heparin and its associated factors can form complexes with PV to initiate cell entry and infection even in HSPG deficient cells.

#### **$\gamma$ -secretase is required for HPV infection after furin cleavage**

We previously reported that PV infection requires  $\gamma$ -secretase and that inhibitors of  $\gamma$ -secretase such as XXI potently block HPV infection both *in vitro* and *in vivo* (31). To determine whether  $\gamma$ -secretase functions before or after furin-cleavage of L2, we assessed the infectivity of HPV16 fcPsV and PsV infection in the presence of XXI both *in vitro* and *in*

*vivo*. XXI inhibited fcPsV and PsV infection similarly, both *in vitro* and *in vivo*, demonstrating that  $\gamma$ -secretase provides a critical function in HPV infection after furin cleavage of L2 (Figure 11E, F).

### **L2 exposure is enhanced in HPV16 fcPsV but it remains immunologically subdominant**

The majority of L2 is buried below the capsid surface of mature virions, including residues 17-36 that are recognized by the cross-neutralizing monoclonal antibody RG-1 [68]. However, conformational changes in the capsid and furin cleavage of L2 during infection render the RG-1 epitope accessible once HPV16 adopts its intermediate structure. To determine if fcPsV also have this property, we performed immunoprecipitation of equal amounts of purified HPV16 PsV or fcPsV with the RG-1. RG-1 was able to pull down a significant amount of HPV16 fcPsV but not PsV (Figure 12A) indicating that furin cleavage during fcPsV production enhanced the exposure of L2 17-36 region upon the capsid surface, consistent with an intermediate conformation. Importantly, since fcPsV highly displays the L2 17-36 region (RG-1 epitope) on the capsid surface, whereas mature PsV do not (Figure 12A), we examined whether L2 in fcPsV was still immunologically sub-dominant to L1. Three groups of ten mice were vaccinated three times each at two week intervals with PBS, HPV16 PsV or fcPsV particles respectively, each formulated in alum and MPL adjuvant. Two additional groups of ten mice were also included in this study; one group was not vaccinated, while the other group was vaccinated with 0.1x of a human dose of Cervarix. Subsequently, full length HPV16 L2 peptide or HPV16 PsV ELISA studies were performed on sera obtained two weeks after the second vaccination or two weeks post the final vaccination. As a positive control for L2 ELISA responses, sera from a previous study with 10 mice vaccinated with L2 $\alpha$ (11-88)X8 was used [8]. These ELISA studies showed a similarly robust HPV16 L1 VLP-specific antibody response in sera of mice vaccinated with PsV, fcPsV or Cervarix after either two or three doses. However, negligible L2-specific antibody levels were detected L2-specific ELISA in either group, suggesting that L2 remains subdominant to L1 in fcPsV despite its surface display of the 17-36 epitope (Figure 12B).

### **Use of fcPsV to enhance detection of L2-specific neutralizing antibodies**

The ability to effectively detect L2-specific neutralizing antibodies is critical to the development of several second generation HPV vaccines. However considerable evidence suggests that the standard *in vitro* HPV PsV neutralization assay (now termed as 293TT- or L1-assay) developed by Pastrana and colleagues [254] is highly effective for L1-specific responses, but lacks sensitivity for L2-specific neutralizing antibodies. This lack of sensitivity was attributed to the failure of the 293TT assay to replicate the spacial-temporal separation between engagement with the primary and secondary receptors which limits the exposure of the dominant L2 cross-neutralizing epitopes (e.g. L2 17-36) to the antibodies . As the purified fcPsV already displays L2 17-36 on its surface, and we hypothesized that its use in the 293TT assay might therefore enhance sensitivity for the detection of L2-specific neutralizing antibodies without compromise of L1-specific measurements. Surprisingly, the substitution of HPV16 fcPsV into the framework of the existing 293TT assay failed to significantly increase the sensitivity of the assay (data not shown).

Studies have previously showed that HPV infection occurs whereby virions can bind directly to 293TT cells and readily enter the cell, thus avoiding the extended display of L2 by ECM-bound virus and limiting opportunity for neutralization by L2 antibodies in the milieu. Further, remaining uncleaved PsV in the fcPsV preparations can still infect 293TT cells, and are possibly cleaved by furin after their rapid uptake, rendering them resistant to L2-specific neutralizing antibodies. To eliminate the latter issue, 293TT cells were substituted with a furin-deficient line, FD11, as the target cell. While this approach (termed ‘FD11 assay’) was more sensitive in detecting L2-specific antibodies that neutralize HPV16 (Table 3), the lack of the T-antigen in FD11 resulted in the FD11 assays for several other HPV genotypes such as HPV18 PsV to display poor reporter signals. This resulted in difficulty establishing accurate 50% neutralization values. Furthermore, FD11 originates from hamster cells (CHO cells), and a human target cell would potentially be more appropriate.



To improve infection sensitivity while maintaining a requirement for furin cleavage, we decided to create a human cell line for detecting HPV neutralization via stably expressing the SV40 T-antigen (T-Ag) protein in LoVo cells, a human adenocarcinoma cell line that is furin-deficient and strongly expresses ECM. This new cell line, called LoVoT, retained the susceptibility for infection by fcPsV. Further, the LoVoT cells express the SV40 large T-antigen that provides a higher reporter signal compared to FD11 or the parental LoVo cells suggesting that a lower viral inoculum can be utilized (Figure 13A-B). Importantly, we assessed several L2-specific sera and monoclonal antibodies using LoVoT as the target cell line for HPV *in vitro* neutralization assays (termed ‘LoVoT assay’) against furin-cleaved PsV of HPV types 16, 18, 31, 45, 58 and 6, in all cases, the detection sensitivity towards L2-based neutralization titers for these HPV genotypes was higher by at least 10-300 fold compared to titers detected by the 293TT assay (Table 4).

The LoVoT assay also demonstrated a similar sensitivity for HPV 16 (60,367, CI=52,999 to 68,760 for 293TT versus 133,179, CI= 54,345 to 326,373, for LoVoT) and HPV18 (512,462, CI=459,833 to 571,115 for 293TT versus 419,401, CI=284,189 to 615,995 for LoVoT) L1-specific neutralizing antibodies induced by vaccination of mice with Cervarix, the licensed L1-based vaccine made from HPV16 and HPV18 VLPs. Likewise, the LoVoT assay displayed similar sensitivity towards HPV6 L1-specific neutralizing antibodies elicited by Gardasil (92,623, CI= 71,929-119,271 for 293TT versus 73,113, CI=49,166-108,723 for LoVoT) (Table 2). Importantly, cross-neutralizing titers were detected with the LoVoT assay against both HPV31 and HPV45 in serum from mice vaccinated with Cervarix but the 293TT assay only detected low titers from HPV31 (3,331, CI=1859-10,547 for 293TT versus 4,428, CI=1,459-7,604 for LovoT) but not for HPV45 (<50 for 293TT versus 1712, CI=917-3194 for LoVoT,). We have shown previously that vaccination with Cervarix protects mice from vaginal challenge with HPV31 and HPV45 PsV [8], and this is also consistent with human data from clinical vaccine trials (56). These results suggest that our new format neutralization assay using fcPsV and the LoVoT line as target cells is a more sensitive assay compared to

the conventional 293TT neutralization assay for detection of L2-specific and cross-neutralizing L1-specific antibodies while retaining the standard assay's high sensitivity for type-specific antibodies to L1 VLP, simplicity and throughput.

## **DISCUSSION**

L2 17-36 epitope exposure occurs as early as four hours after addition of virus to cultured cells, and the infectious process is slow such that addition of antibody even 8 hours later is still neutralizing [45, 274], implying that HPV can exist in its secondary furin-cleaved conformation at the cell surface for several hours. This important insight may help explain why prophylactic HPV vaccination is so effective, and it likely impacts the measurement of L2-dependent neutralizing antibodies. In light of this, our ability to purify >80-90% furin-cleaved HPV PsV particles at milligram titers using our newly created cell line 293TTF provides the opportunity to study both basic and translational aspects of HPV in its furin-cleaved intermediate conformation in areas including structure, biology and immunology.

As expected, characteristics of furin-cleaved particles include furin-independent infection (Figure 8B-E). While some inhibition was noted in the infectivity of HPV fcPsV16 in 293TT cells in the presence of furin inhibitor (Figure 8E), we showed that this inhibition was due to the presence of uncleaved virions in the fcPsV preparation. Further, we showed that optimizing the conditions during the virion maturation period to enhance L2 cleavage will in turn increase the ratio of fcPsV to PsV and promote higher furin-independent infectivity (Figure 9 and 14). Given that fcPsV particles have already bypassed the rate-limiting furin cleavage step, they should also theoretically infect cells with a more rapid time course, and we observed evidence for this at early time points of 24 and 48 hours post infection. However the difference has narrowed by 72 hours post addition of virus to 293TT cells. Similarly, the *in vivo* infectivity of PsV and fcPsV was not significantly different at 72 hours post challenge (Figure 9C-D), suggesting sufficient time for HPV PsV to 'catch up' with the wave of fcPsV infection, as supported by our time-based observations in Figure 9C. Immuno-precipitation

studies also showed that the L2 epitope for RG-1 is more exposed upon fcPsV than PsV (Figure 12), demonstrating that their capsid conformations are distinct but the morphological changes are too subtle to be resolved upon negative staining and transmission electron microscopy (Figure 9A-B). Taken together, these results further demonstrate that fcPsV have distinct properties compared to pre-infectious HPV virions (e.g HPV PsV) and are consistent with HPV in an infectious intermediate state that is characterized by the furin-cleaved L2 and has RG-1 epitope exposed.

Studies on the mechanisms and neutralization of HPV infection using PsV or virions derived from organotypic raft culture are occasionally conflicting, which might potentially reflect differences in their extent of furin L2 cleavage. Published studies of the state of PV L2 in clinical samples showed L2 sometimes existing in a doublet [25, 26, 28, 29, 126] which might be consistent with furin cleavage rather than degradation. However, the *in vivo* significance of this doublet is controversial since in other studies, a single band of L2 is observed [27, 40, 55, 275, 276]. This raises the possibility that the fully matured infectious form of papillomaviruses that is shed from the infected lesion is furin-cleaved. Here, our study of L2 in PV virions isolated from several human and animal papillomas did not detect any evidence to suggest the presence of furin-cleaved L2 (Figure 10). Although multiple sized bands of L2 were seen in certain blots (Figure 10C-D), the migration pattern of the lower bands did not correlate with the expected 8-9 amino acid loss in which furin-cleaved L2 would display, as seen in our furin cleavage experiments of purified PsV versus fcPsV in Figure 9E or 9F. Thus, the additional L2 bands observed are probably due to L2 degradation that occurs during the extraction and/or purification process. Importantly, our findings support the validity of the HPV pseudovirus system and that the furin-cleaved form of PV virions is an infectious intermediate rather than the fully mature infectious form.

Carrageenan has shown promise clinically as a vaginal microbicide for protection against sexual transmission of HPV [262]. While studies using purified HPV PsV show convincingly

that carrageenan is a potent inhibitor of infection by uncleaved PsV of various HPV types both *in vitro* and in mice [18, 261], it cannot be completely ruled out that a small proportion of HPV virions upon natural release is furin-cleaved (but not detected by our Western blot analysis). Hence, it is important to assess if L2 cleavage by furin renders this furin-cleaved form of HPV resistant to inhibition by heparin or carrageenan. Given that fcPsV is able to infect HSPG-deficient cell lines, we rationalized the possibility of fcPsV overcoming heparin or carrageenan inhibition. Surprisingly, our studies both *in vitro* and *in vivo* using fcPsV demonstrated that the inhibitory potency of carrageenan or heparin was not compromised in HPV16 if L2 is cleaved (Figure 11A-D). Interestingly, infection was also enhanced at low concentrations of carrageenan or heparin when infectivity was tested in the HSPG-deficient PGSA-745 cells (Figure 11C). However, *in vivo*, the basement membrane where HPV initially binds to is not HSPG-deficient and thus this phenomenon (Figure 11C) probably occurs *in vitro* only. This indicates that carrageenan remains as a promising topical HPV microbicide candidate.

Recently, a study conducted by Richards and colleagues identified two intermediate conformational states prior to infectious entry. The first intermediate state is achieved after primary binding to HSPG specifically at Lys278 and Lys361 on L1 and this can be characterized by L2 exposure and furin-cleavage. The characteristics of the second intermediate state remains ill-defined but is achieved upon the L2-cleaved virion performing secondary interactions with HSPG via L1 at other specific sites. Conforming to the second intermediate state was found to be important for subsequent internalization and viral uncoating [273]. It is tempting to speculate that fcPsV adopts the first intermediate state postulated by Richards et al. This speculation however contradicts the observation that fcPsV can infect cultured cells that lack HSPG such PGSA-745 cells since further secondary conformational changes are required for subsequent internalization and uncoating. However, as mentioned earlier, these “secondary conformations” remains ill-defined requiring specific mutations on L1 for recognition. Hence, it remains difficult to assess whether fcPsV has

undergone these secondary changes. In addition, there is growing evidence that HPV can utilize non-HSPG receptors such as Laminin 332 (L332) for cell binding and infection. In light of this, it is possible that certain interactions might occur between L322 and fcPsV in PGSA-745 cells which could result in secondary conformational changes (induced by L322) that leads to a certain level of infection. This also goes in line with our results (Figure 11C) which showed that small amounts of heparin or carrageenan improved infection in the same cell line, suggesting that the low amounts of HS or carrageenan moieties could induce alternative secondary conformational changes in fcPsV which in turn enhance infection but at high concentrations result in steric hindrance.

In another previous study also, Kines *et al* showed that productive infection of furin-precleaved virus (FPC) in mice still requires basement membrane binding to HSPG. While FPC could also bind to the surface epithelial cells, no infection was seen. This was later linked to the fact that successful HPV infection requires cell division (induced by wounding) and surface epithelial cells in the mouse vagina do not undergo this. In contrast, HSPG-bound virions in the basement membrane would more likely encounter a migrating basal keratinocyte that would subsequently undergo cell division (Kines *et al.*, 2009). Importantly, it was postulated that this prolonged exposure of basement membrane HSPG-bound FPC virion is probably in the first intermediate state and would have subsequently undergone a second round of conformational changes that allows it to bind to the secondary receptor on the basal keratinocyte. In light of these two findings, it is possible that fcPsV in the presence of heparin or carrageenan was non-infectious *in vivo* due to the presence of saturating amounts of heparin or carrageenan on the HSPG-bound virion. This in turn resulted in steric hindrance exerted against the secondary conformational changes which in turn prevents direct engagement with the secondary receptor basal keratinocytes.

Our results using fcPsV also further elucidated carrageenan's mechanism of action. Previously, Buck and colleagues suggested that the primary mechanism of inhibition is similar to heparin blocking the initial interaction between HSPGs and the viral capsid. Further, it was shown that carrageenan had a secondary inhibitory effect that was exerted after the virions were cell-bound. This secondary inhibition was termed as 'HSPG-independent inhibition' because it was reported using 50-fold more HPV PsV pre-bound to PGSA-754 cells which do not have HSPG. However, the exact mechanism of this secondary inhibitory mechanism was unclear, although two possible modes of actions may be considered. Either the secondary inhibitory effect could occur via preventing the interaction of HPV capsid with the unknown secondary receptor necessary for the remainder of the infectious process or carrageenan prevents the cell-bound HPV pseudovirus from further conformational change to initiate furin cleavage [261]. Given that furin-cleaved HPV has most likely already undergone the first round of conformational change which includes surface display of the amino-terminus of L2, our results here suggest that the secondary inhibitory mechanism of carrageenan is most likely the masking of virus surfaces involved in binding to the secondary receptor and not the prevention of initial conformational changes of the capsid that lead to L2 exposure and furin cleavage. This may also explain why carrageenan is a more potent inhibitor of HPV compared to heparin, as the latter only inhibits the interaction with the primary receptor.

We recently discovered  $\gamma$ -secretase inhibitor XXI is a potent inhibitor of HPV infection. While XXI prevents the escape of the viral DNA from the endosome, the proteolytic target for  $\gamma$ -secretase and how it relates to HPV infection remains unknown. Given that furin cleavage is a necessary intermediate step in infection, the inhibition of HPV16 fcPsV infection by XXI both *in vitro* and *in vivo* (Figure 11E-F) demonstrates that  $\gamma$ -secretase acts after furin during HPV infection.

Natural PV infection frequently triggers an antibody response to L1, but rarely to L2. Likewise, vaccination with virions or L1/L2 VLP induces a potent L1-specific response, whereas minimal titers are observed to L2, whereas vaccination with L2 alone does induce a strong L2-specific antibody response. The subdominance of L2 in the context of the capsid may reflect its low stoichiometry L1 ( $\leq 1:5$ ) and wider spacing compared to L1, and that L2 is predominantly buried below the capsid surface [198]. Indeed, L2 epitope display by insertion into an immunodominant epitope of the major capsid antigen for HPV and non-HPV VLPs does enhance its immunogenicity [10, 201]. In contrast, fcPsV particles display the L2 17-36 epitope with an untethered end in the ordered array of the capsid surface [30]. Nevertheless, no significant L2 antibody titer was detected after vaccination with fcPsV despite a robust L1-specific response (Figure 12B). The continued dominance of L1 responses for fcPsV may reflect both the  $\geq 5:1$  ratio of L1 to L2 in the capsid, and greater spacing between the L2 versus L1 epitopes. Further, since the amino terminus of L2 contains cross-type protective epitopes, we speculate that the subdominance of this region conveys evolutionary advantage by preventing a singly infected host from developing broad immunity to other PV infections.

L2 has received attention as a target antigen for second generation HPV vaccine development because of its potential to induce broad immunity. Clinical development of HPV vaccines requires an effective immune correlate. Given the central role of neutralizing antibodies in protection, it is critical to have an *in vitro* neutralization assay to measure the relevant immune correlate. While the classical PsV-based neutralization assay (293TT assay) has proven a useful correlate for L1 VLP vaccines, it is insensitive for detection of L2-specific neutralizing antibody and also cross-protective L1 responses in weaker sera. Recently an L2-specific neutralization assay has been described [277]. This involves creating a basement membrane-like environment that generates furin-cleaved virions before the neutralization assay is carried out. Briefly, an ECM derived from a breast cancer cell line is first laid down before adding virions. Virion interaction with this environment triggers conformational

change leading to exposure of L2. Supernatant from a separate furin secreting cell line is subsequently added to exert furin cleavage. Lastly, a heparin-deficient CHO cell line, PGSA-745 is added to the mixture as the targeted host cell and expression of the reporter GFP is detected by flow cytometry. While the assay has proven to be superior to the classical 293TT/L1-assay, several recognized limitations exist. Firstly, several cell lines need to be maintained and from a quality control perspective, it is also technically challenging to ensure consistently that equivalent amounts of furin from a cell supernatant is added each time. Secondly, PGSA-745 cells are of non-human origin and while the lack of HSPG in PGSA-745 will limit the infectivity to only furin cleaved virions, the absence of T-antigen in these cell lines suggests similar problems in reporter signal activity would be experienced with “weaker” HPV PsV.

To circumvent such issues and based upon the concepts illustrated by Day et al [277], we have developed an alternative high through-put neutralization assay via the use of fcPsV. Importantly, the fcPsV approach uses a format analogous to the classical 293TT assay. It is based upon the neutralization of fcPsV infection of a furin-deficient human cell line stably expressing the SV40 T-antigen (LoVoT). This fcPsV assay exhibits significantly improved sensitivity in measuring neutralization by L2-specific antisera and monoclonal antibodies by 10-300 fold, and yet maintains similar sensitivity for type-restricted responses to L1 VLP (Table 4).

Vaccination with Cervarix confers protection against HPV 31 and 45 infection in both people and mice [176, 278]. However, using the 293TT assay, no cross-neutralization titers for HPV45 were detected in the sera of mice vaccinated with Cervarix although cross-neutralization titers were detected for HPV31. The use of the LoVoT assay however revealed similar levels of neutralizing titers against HPV31 in sera from mice vaccinated with Cervarix and even detected the presence of low titer neutralizing antibodies to HPV45 (Table 4). This ability to better detect low titer cross-neutralizing L1 antibody titers suggests that the



conformation of fcPsV may better display these cross-protective L1 epitopes than mature PsV. Taken together, these results indicate that the LoVoT assay not only detects L2-specific antibodies with enhanced sensitivity, but potentially also cross-neutralizing L1-specific antibodies, without compromising the measurement of type-specific antibodies to L1 VLP (Table 4).

In summary, our results support the utility of fcPsV system to study several biological and immunological properties of HPV while in its furin-cleaved intermediate conformation. In addition, the LoVoT-based neutralization assay has promise as a tool in HPV vaccine development, especially for evaluating L2-specific and L1-cross neutralization.

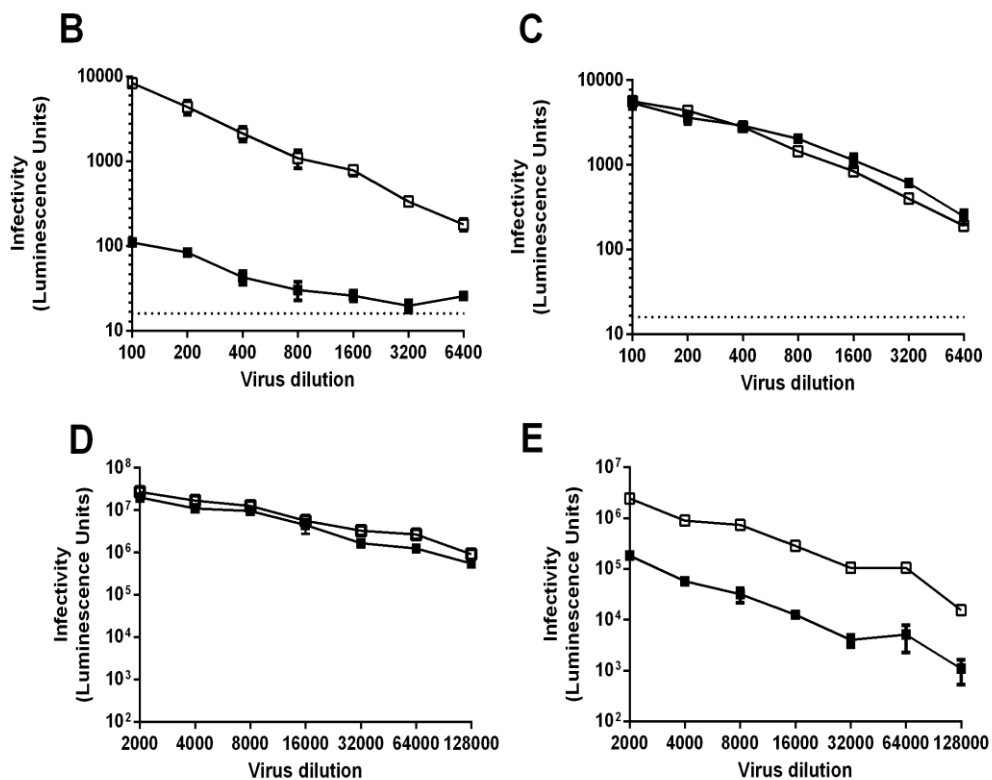
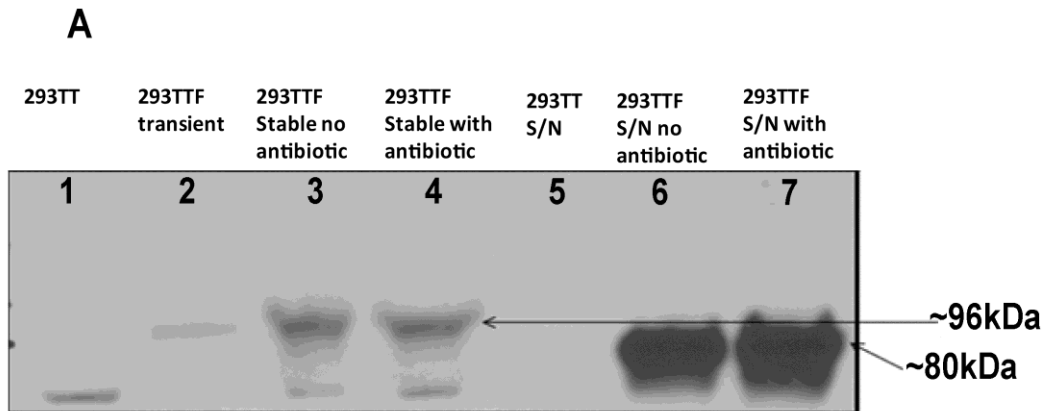
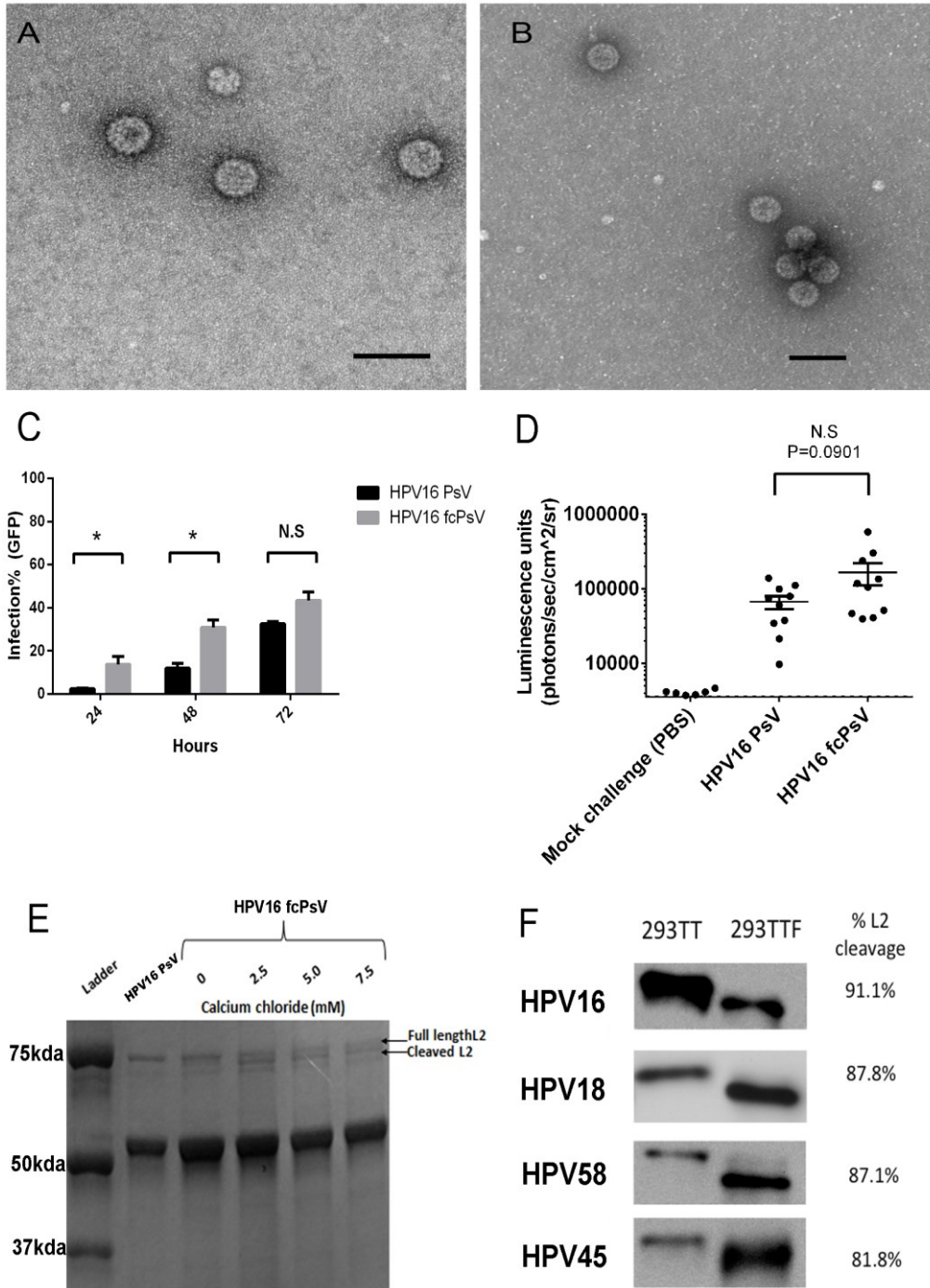


Figure 8. **293TTF cell line overexpresses furin and facilitates production of furin-cleaved PsV.** Expression of endogenous furin in parental 293TT cells (lane 1), 293TT transiently transfected with furin gene (lane 2), 293TTF cells stably transfected with furin in the presence (lane 3) and absence (lane 4) of puromycin (2 $\mu$ g/ml). Equivalent amounts of conditioned media supernatant of parental 293TT (lane 5) or 293TTF in the presence (lane 6) and absence (lane 7) of puromycin (2 $\mu$ g/ml) were used to assess furin secretion. Image analyses and relative densitometry using ImageJ indicate that for 293TTF cells endogenous furin expression and secretion was at least 150-fold higher compared to 293TT control cells.

Band seen at ~60kDa was a furin splice variant as indicated in the material data sheet of the antibody used (A). Equivalent amounts of HPV16 PsV (solid squares) or HPV16 fcPsV (open squares) based on L1 content, were added onto pre-plated furin deficient cells FD11 (B) or FD11F (FD11 cells re-complemented with furin gene) (C), 293TT cells (D) and 293TT cells with 20 $\mu$ M of furin inhibitor (E). All experiments were performed in triplicate



**Figure 9. Furin-cleaved pseudovirus (fcPsV) exhibits 80-90% L2 cleavage without compromise of morphology or infectivity in vivo.** Transmission electron microscopy of HPV16 PsV (180000x) (A) and fcPsV (135000x) (B), black bar indicates 100nm. Infection time course of HPV16 PsV versus fcPsV encapsidating a GFP reporter was added to 293TT cells (60,000 cells/well) (significance was calculated using paired t-test: 24 hours P=0.0349,

48 hours  $P=0.0282$ , 72 hours  $P=0.0654$ ) (C). In vivo vaginal challenge of mice for 72 hours with HPV16 PsV or fcPsV encapsidating a luciferase reporter (unpaired t-test,  $P=0.0901$ ), or PBS as negative control (D). Commassie gel showing L2 cleavage status of HPV16 PsV using the standard PsV protocol or in 293TTF (fcPsV) matured in various calcium concentrations (0, 2.5, 5.0 and 7.5 mM) for 24 hours (E) Western blot analysis of L2 cleavage on HPV PsV genotypes 16, 18, 45, 58 made in either in 293TT with the standard HPV PsV protocol or in 293TTF with modified maturation timing of 48 hours and 5mM of calcium chloride (F)

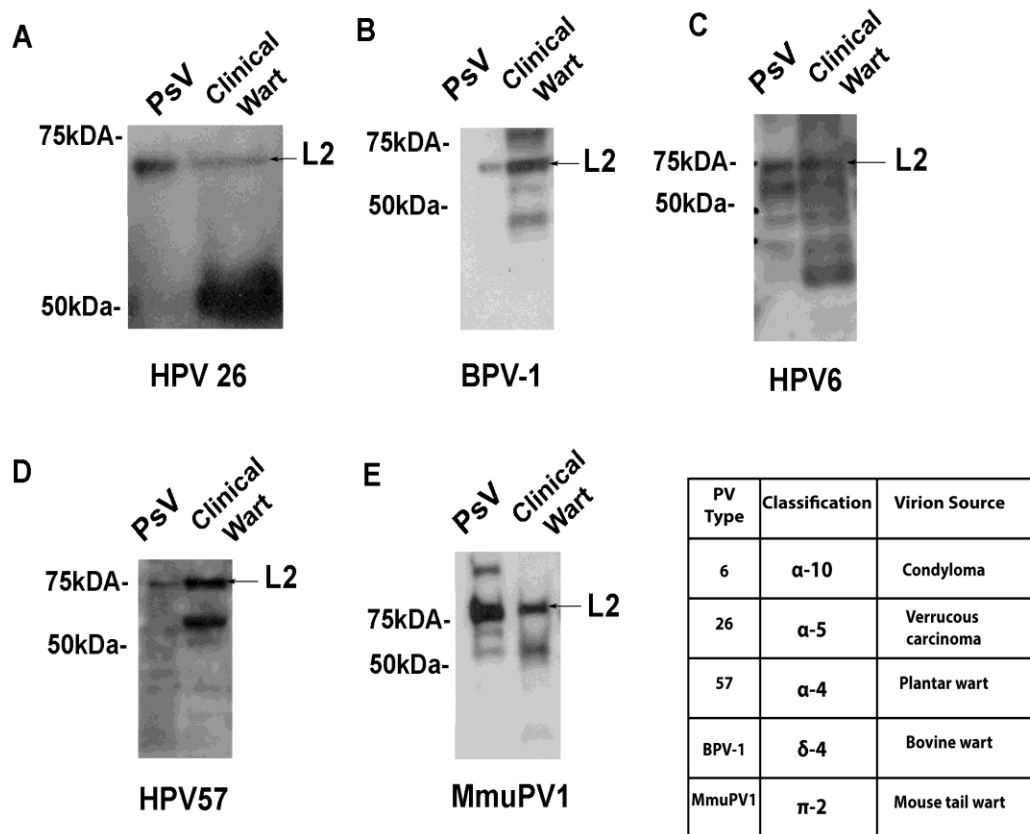


Figure 10. Absence of furin-cleaved L2 from papillomaviruses of natural isolates.

Western blot of papillomavirus pseudovirions (PsV) and PV virions L2 extracted from clinical isolates of warts of the same papillomavirus genotype; HPV26 (A), BPV-1 (B), HPV6 (C), HPV57 (D), MmuPV1 (E) and table showing classification and clinical source of respective papillomavirus.

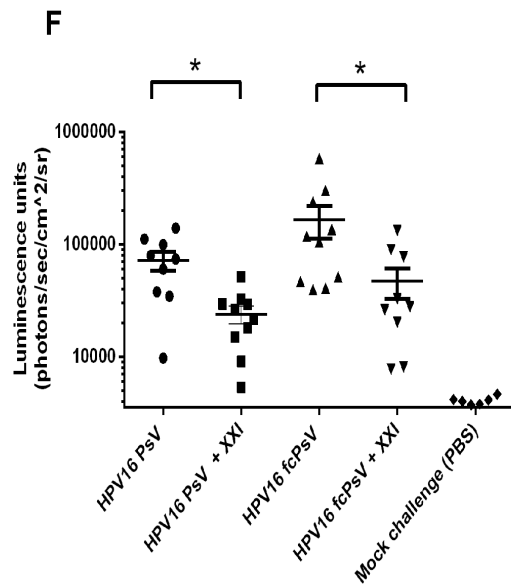
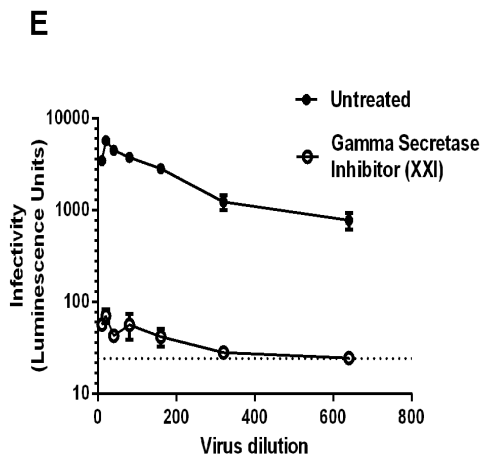
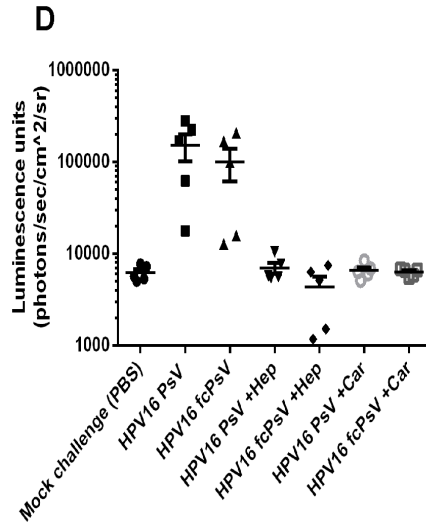
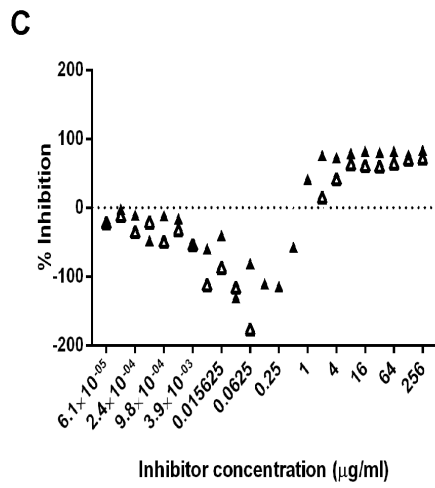
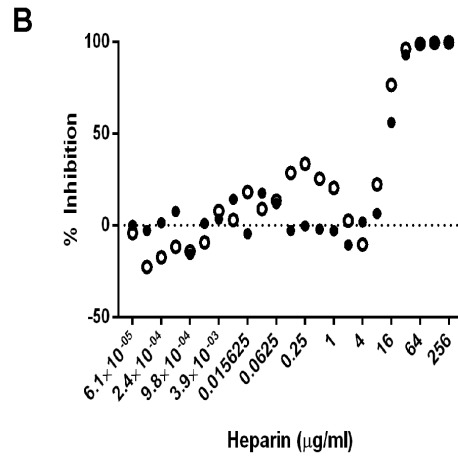
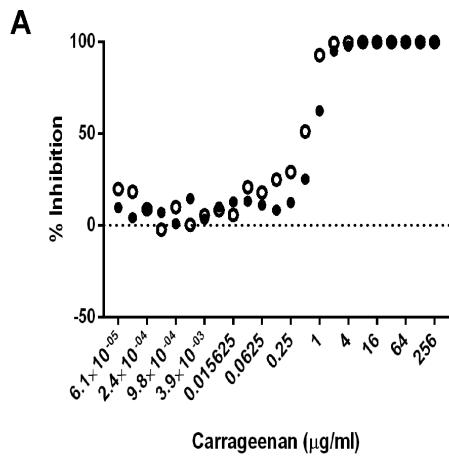


Figure 11. **Heparin, Carrageenan and  $\gamma$ -secretase inhibitor (XXI) inhibit both HPV16 PsV and fcPsV.** Inhibition assays were performed using equivalent amounts of HPV16 PsV (solid circles) or fcPsV (open circles) based on L1 content with varying concentrations of heparin (A), carrageenan (B) in 293TT cells. The same assay was performed using fcPsV16 infection of HSPG-deficient PGSA-745 cell line in the presence of either heparin (open triangles) or carrageenan (closed triangles) (C). In vivo mouse (n=5) vaginal challenge of HPV16 fcPsV or PsV in the presence or absence of heparin or carrageenan (D). Inhibition assay using HPV16 fcPsV in the presence or absence of XXI (500nM) in FD11 cells (furin-deficient CHO cells) (E). In vivo mouse (n=10) challenge with HPV16 fcPsV or PsV in the presence or absence of XXI. \* indicates significance (P value= 0.0089 for HPV16 PsV comparison, P value= 0.0101 for HPV16 fcPsV comparison).



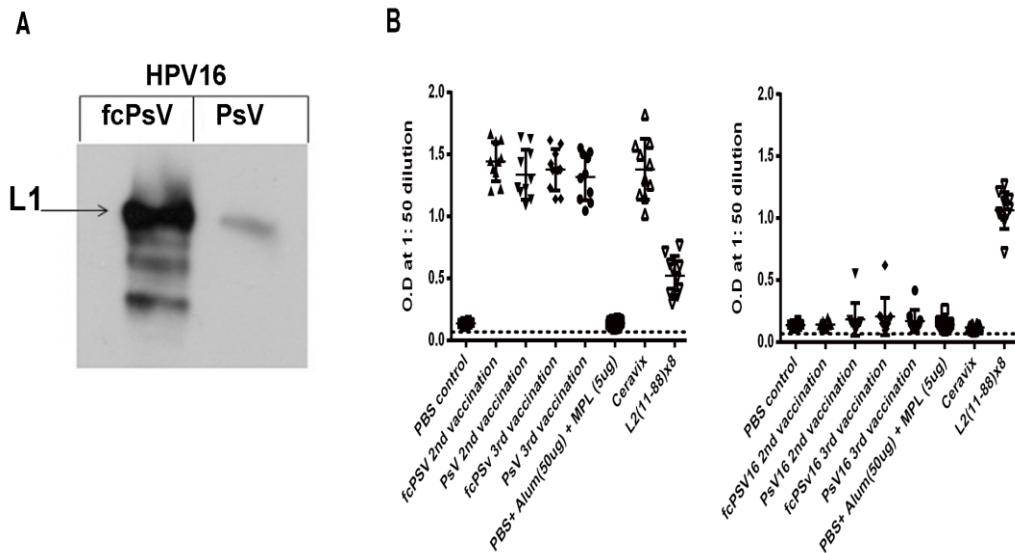


Figure 12. Vaccination with HPV16 fcPsV particles which have enhanced RG-1 epitope exposure does not elicit an enhanced L2 immune response compared to PsV.

RG-1 Immunoprecipitation of equivalent amount of HPV16 fcPsV and PsV (based on L1 content) and immunoblot with antibody to L1. The arrow indicates amount of L1 pulled-down (A). ELISA results for mouse sera vaccinated with HPV16 PsV or fcPsV (plus alum-MPL adjuvant) using HPV16 PsV or full length HPV16 L2 peptide as antigens. (B)

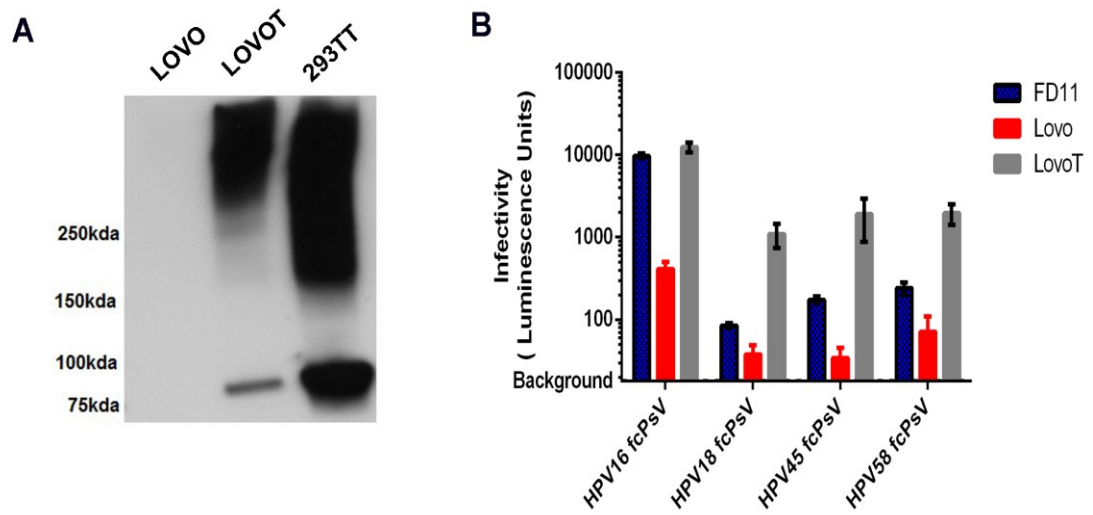


Figure 13. **Generation of LoVoT cell line.** Stable expression of the SV40 T-antigen in LoVoT (lane 2) with parental LoVo cells (lane 1) and 293TT (lane3) as controls (A).

Validation of LoVoT cell line demonstrating superior infectivity (luminescence signal) for the same input of HPV16 fcPsV using virus dilution of 1:8000 (B).

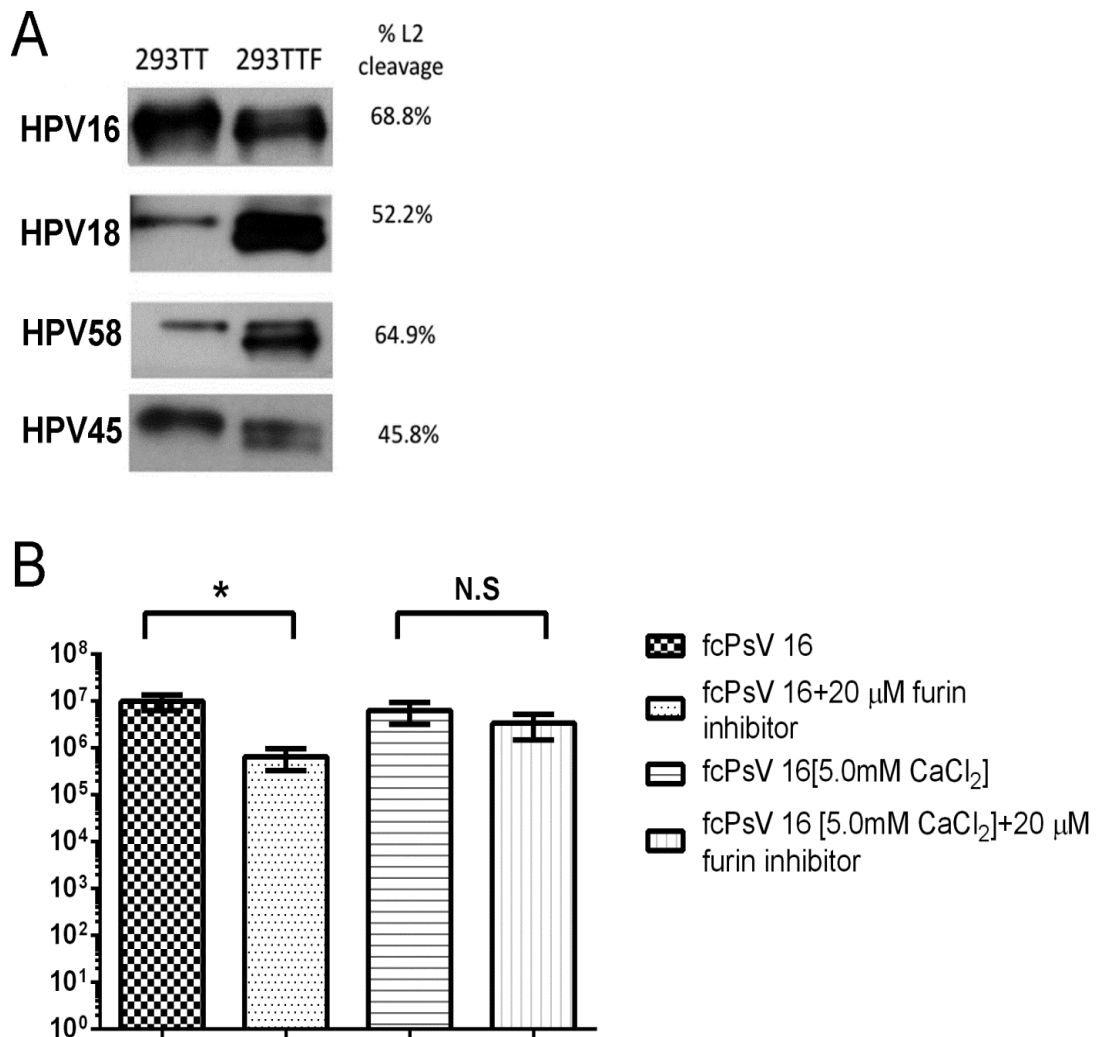


Figure 14. **Optimization of the maturation conditions in the pseudovirus standard**

**production protocol results in more HPV fcPsV virions.** Western blot analysis of L2

cleavage on HPV PsV genotypes 16, 18, 45, 58 made in either in 293TT or 293TTF using the standard HPV PsV protocol methodology shows approximately 50% of L2 being cleaved

only (A). Infectivity test comparing equal amounts of HPV16 fcPsV (labeled fcPsV16) made with the standard protocol conditions or the standard protocol with optimized maturation

conditions in the presence or absence of furin inhibitor (20 $\mu$ M). Experiments were performed

in triplicate and significance was calculated using the Mann-Whitney Test (B)

**Table 3. Summary of neutralization assay titers against HPV16 comparing the conventional HPV16 PsV neutralization assay and using fcPsV in FD11 cells.** Mean neutralization titers based on the reciprocal of the dilution that causes 50% reduction in luciferase activity is recorded. P-value is based on Wilcoxon matched-pairs signed rank test.

<b>Serum/monoclonal antibody used (replicates)</b>	<b>293TT assay neutralization titers (mean)</b>	<b>FD11 assay neutralization titers (mean)</b>	<b>Approximate fold change</b>	<b>P-value</b>
L2 $\alpha$ (11-88)x5 (n=8)	788	8150	10	0.0078
L2 $\alpha$ (11-88)x8 (n=10)	1270	9480	11	0.0156
Mouse monoclonal antibody, RG-1 (n=10)	320	88320	276	0.0020
Rat monoclonal antibody, WW1 (n=10)	70	76800	1097	0.0020
Cervarix <sup>TM</sup> (n=10)	228280	143360	-1.5	0.1055

**Table 4 (over). Summary of neutralization assay titers with a variety of HPV genotypes comparing the conventional 293TT neutralization assay and the new LoVoT assay.**

Mean neutralization titers is based on the reciprocal of the dilution that causes 50% reduction in luciferase activity. Serum pooled from 10 mice vaccinated with either L2 $\alpha$ (11-88)x5 or L2 $\alpha$ (11-88)x8 or L2-specific monoclonal antibodies (MAb) was used by triplicate testing. These antisera were obtained from animals in which vaccination was previously shown to confer protection against *in vivo* mouse challenge with HPV6, 16, 18, 31, 45 and 58.

HPV Virus Studied	Serum/monoclonal antibody used	293TT assay neutralization titers Mean [95% Confidence Interval]	LoVoT assay neutralization titers Mean [95% Confidence Interval]	Approximate fold change (LoVoT titer/293TT titer)
HPV 16 (Luciferase Reporter)	L2 $\alpha$ (11-88)x5	1,236 [944.3-1,617]	13,893 [4,920-39,266]	11.2
	L2 $\alpha$ (11-88)x8	1,252 [865.4-1,812]	31,609 [15,732-63,509]	25.0
	Mouse MAb, RG-1	188 [134-262]	20,638 [15,653-27,210]	110
	Rat MAb, WW1	3,978 [2,784-5,684]	32,768 [17,147-62,619]	8.0
	Cervarix <sup>TM</sup>	60,367 [52,999-68,760]	133,179 [54,345-326,373]	2.2
HPV 18 (Luciferase Reporter)	L2 $\alpha$ (11-88)x5	380 [314-462]	9,229 [4,791-17,778]	24.2
	L2 $\alpha$ (11-88)x8	4,685 [3,963-5,539]	52,158 [56,117-110,171]	11.1
	Mouse MAb, RG-1	1,675 [1,294-2,168]	34,701 [20,650-58,312]	20.7
	Rat MAb, WW1	503 [392-644]	26,209 [16,953-40,519]	52.0
	Cervarix <sup>TM</sup>	512,462 [459,833-571,115]	418,401 [284,189-615,995]	-0.81
HPV 31 (Luciferase Reporter)	L2 $\alpha$ (11-88)x5	<50 [N/A]	305 [109-850]	12.2
	L2 $\alpha$ (11-88)x8	<50 [N/A]	1,438 [670-3,086]	28.8
	Mouse MAb, RG-1	<50 [N/A]	<50 [N/A]	N/A
	Rat MAb, WW1	<50 [N/A]	<50 [N/A]	N/A

	Cervarix <sup>TM</sup>	3,331 [1,459-7,604]	4,428 [1,859-10,547]	1.3
HPV 45  (Luciferase Reporter)	L2α(11-88)x5	52 [29-90]	3,013 [1,746-5,197]	57.9
	L2α(11-88)x8	176 [125-248]	15,497 [10,207-23,530]	87.6
	Mouse MAb, RG-1	<50 [N/A]	<50 [N/A]	N/A
	Rat MAb, WW1	<50 [N/A]	8,033 [4,601-14,024]	321
	Cervarix <sup>TM</sup>	<50 [N/A]	1,712 [917-3,194]	68.5
HPV 58  (Luciferase Reporter)	L2α(11-88)x5	68 [36-126]	4,025 [1,555-10,424]	58.9
	L2α(11-88)x8	416 [286-604]	7,562 [4,788-11,945]	18.2
	Mouse MAb, RG-1	<50 [N/A]	<50 [N/A]	NA
	Rat MAb, WW1	<50 [N/A]	830 [194-3,538]	27.0
	Cervarix <sup>TM</sup>	<50 [N/A]	<50 [N/A]	NA
HPV 6  (Luciferase Reporter)	L2α(11-88)x5	170 [131-220]	2,547 [1,271-5,107]	15.0
	L2α(11-88)x8	288 [227-365]	9,313 [6595-13152]	28
	Mouse MAb, RG-1	<50 [N/A]	<50 [N/A]	NA
	Rat MAb, WW1	<50 [N/A]	<50 [N/A]	NA
	Cervarix <sup>TM</sup>	<50 [N/A]	<50 [N/A]	NA
	Gardasil <sup>TM</sup>	92,623 [71,929-119,271]	73,113 [49,166-108,723]	-1.2

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## **II. Measurement of neutralizing serum antibodies of patients vaccinated with human papillomavirus L1 or L2-based immunogens using furin-cleaved HPV pseudovirions**

### **Abstract**

Antibodies specific for neutralizing epitopes in either Human papillomavirus (HPV) capsid protein L1 or L2 can mediate protection from viral challenge and thus their accurate and sensitive measurement at high throughput is likely informative for monitoring response to prophylactic vaccination. Here we compare measurement of L1 and L2-specific neutralizing antibodies in human sera using the standard Pseudovirion-Based Neutralization Assay (L1-PBNA) with the newer Furin-Cleaved Pseudovirion-Based Neutralization Assay (FC-PBNA), a modification of the L1-PBNA intended to improve sensitivity towards L2-specific neutralizing antibodies without compromising assay of L1-specific responses. For detection of L1-specific neutralizing antibodies in human sera, the FC-PBNA and L1-PBNA assays showed similar sensitivity and a high level of correlation using WHO standard sera (n=2), and sera from patients vaccinated with Gardasil® (n=30) or an experimental human papillomavirus type 16 (HPV16) L1 VLP vaccine (n=70). The detection of L1-specific cross-neutralizing antibodies in these sera using pseudovirions of types phylogenetically-related to those targeted by the L1 virus-like particle (VLP) vaccines was also consistent between the two assays. However, for sera from patients (n=17) vaccinated with an L2-based immunogen (TA-CIN), the FC-PBNA was more sensitive than the L1-PBNA in detecting L2-specific neutralizing antibodies. Further, the neutralizing antibody titers measured with the FC-PBNA correlated with those determined with the L2-PBNA, another modification of the L1-PBNA that spacio-temporally separates primary and secondary receptor engagement, as well as the protective titers measured using passive transfer studies in the murine genital-challenge model. In sum, the FC-PBNA provided sensitive measurement for both L1 VLP and L2-specific neutralizing antibody in human sera. Vaccination with TA-CIN elicits weak cross-protective antibody in a subset of patients, suggesting the need for an adjuvant.



## **Introduction**

The seminal discovery by zur Hausen that certain oncogenic genotypes of Human papillomaviruses (HPV) typified by HPV16 are the etiologic agents of cervical cancer has led to the commercial development of two preventive vaccines, Gardasil® and Cervarix® [279]. Their development began with the demonstration that major capsid protein L1 self-assembles into virus-like particles (VLP) [24]. L1 VLP vaccination elicits high titers of type-restricted serum neutralizing antibodies which confer protection from experimental viral challenge after passive transfer of naïve animals [280, 281]. In line with preclinical studies, vaccination of patients with HPV16 L1 VLP also induces type-restricted neutralizing antibodies, suggesting the need for multivalent formulation [177]. As a result, both licensed vaccines contain L1 VLPs derived from HPV16 and HPV18, the oncogenic genotypes that respectively cause circa 50% and 20% of all cervical cancer cases. Gardasil® also contains L1 VLP of benign genotypes HPV6 and HPV11, which are the most common cause of genital warts. These L1 VLP vaccines were proven safe, highly immunogenic, and protective against infection and anogenital neoplasia associated with the vaccinal genotypes [116, 282-285]. However, these vaccines confer limited cross-protective potential towards the most phylogenically-related types and none for the ~12 other oncogenic HPV types that together cause the remaining ~30% of cervical cancer cases [117, 176, 278]. A nonavalent prophylactic VLP vaccine being developed by Merck is intended to broaden protection against the remaining oncogenic HPV types, but this complex formulation may be costly to produce, limiting access for low resource settings [286].

An alternative approach to broaden protection is vaccination with the papillomavirus minor capsid protein L2 which induces broadly cross-neutralizing antibodies and protects against experimental challenge with diverse HPV genotypes in animal models [126, 198, 287]. Further, L2-based HPV vaccines can be simply and potentially inexpensively manufactured as a single antigen in bacteria. However, L2 is weakly immunogenic in animals compared to L1 VLP [123, 201, 288]. No clinical studies have examined the ability of L2-based

vaccination to protect against natural acquisition of HPV infection, although a few have tested its immunogenicity in patients. For example, the safety and immunogenicity of TA-CIN, a fusion protein of HPV16 E6, E7 and L2 produced in bacteria, has been tested in healthy volunteers and women with high grade vulval intraepithelial neoplasia (VIN), alone or in combination with topical imiquimod or a recombinant vaccinia virus expressing E6 and E7 (TA-HPV) [289-291]. Vaccination with TA-CIN elicited low titers of HPV16 and HPV18 neutralizing antibodies, and the L2-specific antibody responses in VIN patients were significantly lower than for healthy volunteers [292].

Production of native HPV *in vitro* requires specialized culture conditions, and infection does not have a readily discernible phenotype in animals. Hence, HPV pseudovirus production using codon optimized L1 and L2 genes and the encapsidation of a luciferase marker plasmid to facilitate the detection of infection of 293TT cells or upon vaginal challenge of mice have been used to circumvent these limitations[17, 260]. Using these tools, it was shown that active immunization with L2 immunogens or passive transfer of naïve mice with L2 antisera protects against experimental vaginal challenge with HPV pseudovirus. These antisera often have robust L2 ELISA titers but surprisingly, a low or undetectable neutralization titer when assessed with the standard *in vitro* HPV pseudovirus-based neutralization assay (L1-PBNA) [8, 68, 120, 130, 293]. These observations suggest that vaccination with L2 results in predominantly non-neutralizing antibodies [41] and/or that the L1-PBNA is insensitive for the detection of L2-specific neutralizing antibodies.

Day *et al* identified spatio-temporal differences during the early events of HPV infection *in vivo* versus the *in vitro* infection of 293TT cells resulting in an abbreviated opportunity for furin cleavage of L2, exposure of its neutralization epitopes on the virus surface and thus for L2-mediated neutralization to occur in the L1-PBNA [44, 46, 294]. To enhance the sensitivity for L2-specific neutralizing antibodies, improved neutralization assays have been developed recently [11, 13, 295]. Day *et al* utilized an extracellular matrix, exogenous furin and a

different target cell line to ensure L2 neutralizing epitope exposure in their L2-PBNA and better emulate infection *in vivo*. An alternate strategy to improve sensitivity for L2 antisera is the use of a furin cleaved pseudovirion-based neutralization assay (FC-PBNA) in which antibodies prevent HPV furin-cleaved pseudovirions (fcPsVs) from infecting a furin-deficient LoVoT cell line. These HPV fcPsVs are produced from a cell line that over-expresses furin (293TTF) and virion particles produced from this cell line have exposed neutralizing epitopes of L2 [13].

While the L2-PBNA and FC-PBNA have previously shown better sensitivity towards L2-specific neutralizing antibodies in animal sera, they have not been validated using human serum. Here, we examine the utility of the FC-PBNA for high throughput measurement of both L1 VLP and L2-specific neutralizing antibodies in human sera, and examine its correlation to the L1- and L2-PBNAs, and protection from vaginal challenge with HPV pseudovirion upon passive transfer of naïve mice with titrated sera of patients vaccinated with L2.

## **Materials and Methods**

### **Ethics Statement**

Sera of patients vaccinated with Gardasil® (n=30) as part of their routine clinical care were obtained with written informed consent at the University of Alabama, Birmingham and with the prior permission of the University of Alabama, Birmingham Internal Review Board (X081124003). The studies using human sera were done with the prior permission of the Johns Hopkins University Internal Review Board (NA\_00043331). Animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and with the prior approval of the Animal Care and Use Committee of Johns Hopkins University (MO13M425). Mice were anesthetized with isoflurane inhalation and euthanized by carbon dioxide asphyxiation.

### **Generation of HPV Pseudovirus (PsV) and HPV furin-cleaved Pseudovirus (fcPsV)**

A firefly luciferase expression plasmid was employed as the reporter for both HPV PsV and fcPsV. HPV PsV was used in the L1-PBNA and for mouse challenge studies. The HPV fcPsVs were used for FC-PBNA. Standard PsV were generated in 293TT cells as described in the protocol (<http://home.ccr.cancer.gov/Lco/pseudovirusproduction.htm>). For HPV fcPsV, the methodology was employed as described in [13]. Following virus generation and purification, respective virus fractions were serially diluted and tested on 293TT or LoVoT cultures to assess reporter gene expression and determine the highest dilution value prior to reporter signal saturation. This dilution value was used for all subsequent infectivity and neutralization assays. If reporter signal was not saturating at 1:1000 (as seen with HPV18 PsV and fcPsV), a general guide line was to choose a dilution where signal was ~100-fold above background. In fcPsV, we determined the acceptable extent of cleavage of L2 confirmed as  $\geq 70\%$  by Western blot analysis.

### **Cell culture and cell lines**

All cell lines were maintained in complete DMEM supplemented with 10% FBS, 1X penicillin/streptomycin, 1X Non-essential amino acids, 1X Sodium Pyruvate (Gibco, Life Technologies, Grand Island NY). 2  $\mu\text{g/ml}$  of puromycin was added to 293TTF cells to maintain furin selection and 200  $\mu\text{g/ml}$  of Hygromycin B was used to maintain LoVoT selection.

### **Human Serum**

Sera of 70/72 patients of which 59 were vaccinated with HPV16 L1 VLP and 11 were placebos were obtained from a completed phase I clinical study [177]. Sera of patients vaccinated with Gardasil® (n=30) as part of their routine clinical care were obtained at the University of Alabama, Birmingham. WHO reference human serum for HPV16 (05/134) and HPV18 (10/140) serology was acquired from NIBSC (Hertfordshire, UK). Sera (n=19) were obtained pre- and one month post-treatment with the topical imiquimod for 8 weeks and 3

doses of 125 µg of HPV16 E6E7L2 (TA-CIN) at monthly intervals in women with high grade vulval intraepithelial neoplasia (VIN) enrolled in a prior phase II trial [296].

### **L1-based Pseudovirus neutralization assay (L1-PBNA), Furin-cleaved based**

#### **Pseudovirus neutralization assay (FC-PBNA) and the L2-PBNA**

Briefly, 293TT cells (for the L1-PBNA) or LoVoT cells (for the FC-PBNA) were seeded at 15,000 cells/well in a 96 well plate. 24 hours later, using another 96 well plate, serum test samples were either serially diluted two-fold (L2 sera at starting dilution 1:50) or three-fold (L1-sera at starting dilution 1:200 for L1 specific antibody assessment and 1:50 for L1 cross neutralizing antibodies assessment) in DMEM culture media, and mixed with HPV PsV (at a dilution previously determined for each batch and type by dilution to ~100x background). In general, approximately 2 to 5-fold more fcPsV was required for the FC-PBNA (e.g 1:5000 HPV16 PsV or 1:1000 HPV16 fcPsV). Mixtures were incubated at 37°C for two hours before being added to 293TT or LoVoT cells. The plates were incubated at 37°C for 72 h. Following this, cells were lysed with 30 µL of Cell Culture Lysis Reagent (Promega, Madison WI) for 15 min at room temperature on a rocking platform. The entire lysates were transferred to a 96-well black plate, and luciferase activity was measured by adding 50µL of luciferin substrate to each well (GloMax<sup>®</sup>-Multi Detection System, Promega, Madison WI). All assays included a neutralizing serum (pooled mouse Gardasil Sera (n=10) for L1 vaccine analysis) and/or L2 monoclonal antibody to assess batch/assay variability and negative control to determine background. For the L2-PBNA, the assay was carried out as per described in <http://home.ccr.cancer.gov/lco/L2neut.htm>

### **ELISA**

Maxisorp microtiter 96-well plates (Thermo Scientific Nunc, Waltham MA) were coated with purified HPV16 full length L2 protein with a 6His tag at 500ng in 100µL PBS/well. The plates were incubated overnight at 4°C and then blocked with PBS/1% BSA for 1h at 37°C. Human serum samples were diluted 1:50 in PBS/1% BSA were then added to the plates in

triplicate for 1h at 37°C. Following this, plates underwent 3 washes with washing buffer (0.01% v/v Tween 20 in PBS) before HRP-sheep anti-human IgG diluted 1:5000 in 1% BSA was added to each well and plates were incubated for 1h at 37°C. After 3 further washes, 100µL of ABTS solution, 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid] (Roche, Basel Switzerland) was added to each well for development, and absorbance at 405nm read using a plate reader Xmark Plus (Bio Rad, Hercules CA).

### **Passive transfer of sera and mouse vaginal challenge studies with HPV58**

Balb/c mice 6-8 weeks old were purchased from Jackson Laboratories (Maine, USA) and were injected subcutaneously with 3mg of medroxyprogesterone (Depo-Provera, Pfizer, New York). Three days later, 100 µL of pre-vaccination patient sera or, 100 µL, 33 µL or 10 µL of post-vaccinated patient sera was injected intra-peritoneally into groups (n=5) of mice. The following day, each mouse was challenged with 2µL of HPV58 PsV in 20 µL ( $2.2 \times 10^9$  Viral Genome Equivalents/mouse). An equal volume of 3% CMC (Carboxymethylcellulose sodium salt, Sigma, St Louis MO, USA) in PBS was added to make a total virus challenge volume of 40 µL per mouse. Mice were anesthetized with isoflurane inhalation to effect prior to administration of virus. Half of the challenge dose (20 µL) was injected into the mouse vaginal vault, followed by insertion of a cytobrush cell collector that was turned both clockwise and counter-clockwise 15 times to induce trauma. After removal of the cytobrush, the remaining half of the inoculum was deposited in the vagina. At 72 h after challenge, the mice were anesthetized again and 20 µL of luciferin (7.8mg/mL) was deposited to in the vaginal vault. Using a Xenogen IVIS 100 imager, bioluminescence was acquired. Signal intensities were further analyzed using Living Image 2.5 software. A mouse plasma volume of 2mL based on previous estimates was used to estimate the dilutions for 10 µL, 33 µL and 100 µL injected human sera as 1:200, 1:60, and 1:20 respectively.

## **Data analysis and Statistics**

For comparisons between all assays, individual human patient sera samples vaccinated with their respective vaccine candidate were analyzed under triplicate L1-PBNA or FC-PBNA assays. To calculate the EC50 value (the reciprocal of the dilution that causes 50% reduction in luciferase activity), the non-linear model  $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC50} - X) * \text{HillSlope}))}$  was fitted to the  $\log_{10}$  transformed neutralization titers triplicate data using Graphpad Prism 6. The estimated EC50 (which is an average of the 3 triplicate studies is reported as the titer. Patients who had neutralization titers of <1:50 were assigned an EC50 value = 1. For assay comparisons, Deming regression [297] and graph plots was applied to the  $\log_2$  transformed observed EC50 values by using R Version 3.03 with package mcr. The error ratio of the two methods was assumed to be 1. The estimation of the regression model parameters, including intercept and slope, and their bootstrap confidence intervals are reported as well as the Pearson's correlation coefficient (r). For L2-PBNA comparisons, the sparse numbers of positive data resulted in confidence intervals being calculated using the jackknife method. All Deming regression values were rounded off to two decimal places, whereas all EC50 values were rounded to the nearest whole number.

## **Results**

### **Sensitivity of FC-PBNA for WHO reference standards to HPV16/18 antibodies**

We first tested the WHO international standards for HPV16 (05/134) [298] and HPV18 (10/140) [299] serology to compare the L1- and FC-PBNA. Our results showed the L1-PBNA and FC-PBNA estimated titers of 120.6 (95% CI = 97.44-149.2) and 146.8 (95% CI=75.33-285.9) respectively for the HPV16 (05/134) serum. Importantly, these titers were within the range (all titers <1:200) described for the WHO HPV16 standard when tested previously by eight independent laboratories. We next tested the WHO HPV18 standard serum using both the L1- and FC-PBNAs and obtained titers of 959.4 (95% CI=840-1095) and 1290 (95% CI=714- 2330) respectively. This EC50 value was also within the range of titers (80-1350) detected by 13 laboratories which previously tested the HPV18 standard. Both standards to 16

and 18 were also tested against different HPV types such as HPV 31 and 45 which resulted in no detectable neutralization at 1:50 dilution (data not shown). Together, this indicates that the L1- and FC-PBNA are similarly sensitive in detecting the HPV16 and HPV18 L1 VLP-specific antibody in the WHO standards which were derived by pooling sera from naturally infected individuals.

### **Correlation of FC-PBNA and L1-PBNA for detection of neutralizing serum antibodies of patients vaccinated with L1 VLPs**

We next compared the L1-PBNA and FC-PBNA using sera from patients vaccinated with HPV16 L1 VLPs or placebo. The first test involved assessing HPV16 neutralizing antibody titers in sera obtained one month following three intramuscular injections of n=70/72 volunteers enrolled in a Phase I clinical trial of an experimental HPV16 L1 VLP vaccine (n=59) or placebo (n=11) [177]. Comparison of methods was performed via Deming regression (see Materials and Methods). Results showed an intercept of 0.29 (95% CI= 0.061,0.67) and a slope of 1.02 (95% CI= 0.97,1.05). In addition, the Pearson's correlation was estimated as  $r= 0.97$  indicating that both methods were very comparable (Figure 15A).

A subset of patients (n=12, including 2 placebos) from this study had been previously tested by Pastrana and colleagues when describing the original L1-PBNA which used the secreted alkaline phosphatase reporter system (SEAP L1-PBNA) instead of firefly luciferase [254]. This further enabled us to compare our FC-PBNA results with the titers described in the published study. Firstly, to check if our L1-PBNA which adopts the luciferase reporter system differed in sensitivity from the SEAP system, we compared our luciferase L1-PBNA detected titers with the published titers obtained using the SEAP L1-PBNA. Deming regression of the intercept and slope was 0.16 (95% CI= -0.048, 6.00) and 1.00 (95% CI= 0.586, 1.05). In addition, Pearson's correlation coefficient was calculated  $r=0.99$  (Figure 15B). Likewise, a Pearson's  $r=0.98$  was found between the FC-PBNA and the SEAP assay tested with the same sera (Intercept = 0.28 [95% CI= -0.08,7.9], Slope = 0.95 [95% CI= 0.43,1.01], Figure 15C).



Taken together, these results show that the FC-PBNA is comparable to the L1-PBNA and that it is similarly as sensitive in detecting HPV L1 specific neutralizing antibodies.

The performance of the L1-PBNA and FC-PBNA in detecting HPV16, HPV18, HPV6 neutralizing antibodies elicited by vaccination was next investigated using sera of patients (n=30) who received Gardasil® as part of their routine clinical care (Table 5). Once again, the results and subsequent Deming regression analysis showed high correlation between both assays indicating that the FC-PBNA is as sensitive as the L1-PBNA for detecting L1-specific neutralizing antibodies in patient sera. HPV16 ( $r=0.96$ , Intercept =0.45 [95% CI=0.06, 1.90], Slope = 1.00 [95% CI= 0.85,1.07]) (Figure 1D), HPV18 ( $r=0.87$ , Intercept =-0.89 [95% CI=-5.09, 0.72], Slope = 0.97 [95% CI= 0.83,1.31]) (Figure 1E) and HPV 6 ( $r=0.93$ , Intercept =0.17 [95% CI=-6.46,1.21], Slope = 1.00 [95% CI= 0.87,1.10]) (Figure 15F).

#### **No difference between assays in detecting L1 cross-neutralizing antibodies against HPV31 and 45 in Gardasil® patient serum**

In our initial FC-PBNA characterization using serum that was pooled from ten Cervarix® vaccinated mice , we reported that our FC-PBNA was potentially more sensitive in detecting L1-VLP cross-neutralizing titers against non-vaccine HPV types 31 and 45 when compared to the L1-PBNA[13]. To further investigate this finding, a similar comparison was performed using the individual human volunteers vaccinated with Gardasil®. The majority of the volunteers failed to mount an *in vitro* neutralization response against these non-vaccine related virus types detectable in either assay (Table 5), although some cross-neutralization was detected against HPV31 and HPV45 (less than 15%). We next looked at the entire data set to assess if patients who responded against either HPV31 or HPV45 typically had higher neutralization titers against HPV16 or HPV18 respectively, but no clear correlation was observed (Table 5). However, because the data set is limited, we cannot exclude this possibility or that at least some of the HPV31 and HPV45 responses were elicited by natural infection with these types.

### **Assay by ELISA and passive transfer of L2-specific antibodies in the sera of patients vaccinated with TA-CIN**

To examine the performance of the neutralization assays for detection of L2-specific antibodies, we utilized pre-immunization and one month post-immunization sera from a phase II study in which 19 vulval intraepithelial neoplasia (VIN) patients who were first treated topically with imiquimod at the lesion site for 8 weeks, and then vaccinated three times at monthly intervals with 125 $\mu$ g of HPV16 E6E7L2 fusion protein (TA-CIN) with no adjuvant [296]. Since the L1-PBNA may not be fully sensitive towards L2 antibodies, we utilized an ELISA assay with full length HPV16 L2 protein for our initial screen (n=17/19 patients due to limiting sera) of both pre-vaccinated and post-vaccinated sera to determine if vaccination elicited L2-specific antibodies. A significant increase in the L2 ELISA was observed post-vaccination in 12 of 17 patients tested (Figure 16A).

As the L2 ELISA cannot determine if the antibody response is protective, we next performed passive transfer studies utilizing the *in vivo* mouse challenge model developed by Roberts and colleagues [18]. Although technically demanding and low throughput, it is currently the most sensitive method for functional antibody assessment [300] and addresses protection from experimental challenge [301]. Importantly, the protective titers determined in these passive transfer studies could subsequently be used for comparison with the *in vitro* neutralizing serum antibody titers detected by the FC-PBNA or L1-PBNA.

As the majority of patients were naturally infected with HPV16 and could therefore have HPV16 L1-specific neutralizing antibodies which can be protective, the passive transfer and PBNA studies were performed with HPV58 pseudovirions. The *in vivo* results (Figure 16B) showed that passive transfer of 100 $\mu$ L of post-vaccinated serum/mouse from 8 out of 17 TA-CIN patients significantly reduced HPV58 infection after vaginal challenge compared to the pre-immunization serum. It is not clear why vaccination with TA-CIN elicited detectable cross-protective antibody responses in only approximately half of these patients, but this is in

line with previous findings that L2 responses in AGIN patients (predominantly VIN patients) vaccinated with TA-CIN were infrequent and of low titer suggesting the requirement for an adjuvant.

### **Correlation between PBNA and protection by passive transfer of patient sera for detection of L2-specific antibodies**

We next sought to compare the *in vitro* PBNA neutralization titers with these *in vivo* protective titers. Thus the TA-CIN patient sera were tested side-by-side with the L1-PBNA and FC-PBNA for *in vitro* HPV58 neutralization titer assessment (Table 6). The L1-PBNA detected neutralizing titers in only 2 out of 17 patient sera at a 1:50 dilution. Conversely, the FC-PBNA was able to detect neutralizing antibodies in more of the patient sera (6 out of 17). Importantly, these 6 patients were within the same 8/17 patient sera which were protective in the *in vivo* HPV58 challenge studies by passive transfer of 100 $\mu$ L, which results in an estimated final dilution of 1:20 in the mouse (Figure 16B).

To further assess analytical sensitivity towards L2-specific neutralizing antibodies, we performed passive transfer studies using titrated amounts of TA-CIN patient sera and correlated the percentage inhibition of HPV58 infection after vaginal challenge to *in vitro* measurements with the FC-PBNA. As the amount of serum for such a study was a limiting factor, only sera from 7 patients could be used. Groups of mice (n=5) were injected intraperitoneally with 10 $\mu$ L, 30 $\mu$ L, or 100 $\mu$ L of post-vaccinated serum or 100  $\mu$ L of pre-vaccinated serum. One day after passive transfer of serum, the mice were challenged with HPV58 pseudovirions. As expected, *in vivo* protection was observed for all five patient sera that were previously positive in both the *in vivo* challenge and FC-PBNA for L2-neutralizing titers. Importantly, the *in vivo* protective titers were very similar in titer value and had overlapping confidence intervals with the *in vitro* neutralizing titers detected by the FC-PBNA (Figure 17, Table 6). In a similar fashion, sera from patients IT-4 and IT-8 which showed an L2-specific response by ELISA but no neutralization titers at 1:50 dilution were

not protective in the titrated passive transfer experiments (data not shown). These findings suggest that the FC-PBNA is able to measure low titers of L2 neutralizing antibodies with greater sensitivity than the L1-PBNA and that its titer measurements were consistent with the protective titers measured using the *in vivo* mouse challenge model.

An *in vitro* PBNA assay with improved sensitivity for L2-specific neutralizing antibodies has been previously reported by Day and colleagues [11]. This L2-PBNA also reported good correlation with passive transfer studies utilizing the *in vivo* mouse challenge model [18]. Using the same sera from patients vaccinated with TA-CIN, we performed the L2-PBNA and compared the titers with those obtained using the FC-PBNA. A Pearson's correlation of  $r=0.93$  (Intercept = 0.22 [95% CI = -0.26, 0.70], slope 1.33 [95% CI = 1.15, 1.51]) was found between these assays suggesting the methods are comparable in sensitivity and the feasibility of using either assay for detection of L2-specific neutralizing antibodies (Figure 17F).

## **Discussion**

In several infectious disease models, low titers of neutralizing antibody titers are sufficient for protection [301]. Indeed patients are durably protected against HPV18 after vaccination with Gardasil despite titers of antibody to the H18.J4 neutralizing epitope measured by cLIA waning to the background cutoff [302, 303]. Importantly, in the majority of these samples neutralizing antibodies could be detected using the L1-PBNA [304], suggesting the importance of a sensitive and functional assay for immune monitoring of prophylactic vaccination. Similarly, Gardasil has been shown to provide cross-protection against HPV31 despite the inability to detect HPV31 neutralizing antibodies in many patients [278]. This suggests that either low levels of HPV neutralizing antibodies are sufficient for protection or that the low titers are coupled with a rapid recall response that is triggered upon infection that then produces sufficient local levels of antibodies in time to provide complete neutralization. Gardasil does elicit a rapid recall response upon intramuscular injection of a fourth dose, but it is important to note that the challenge dose in this study is systemic, and utilizes an

adjuvant and a dose likely far greater than natural viral inoculum exposure at the anogenital epithelium [305] and therefore may not be a true reflection of a recall response to natural challenge. Furthermore, this recall response was measured at 1 week post-inoculation [305] and, although the papillomavirus infectious process is slow, post-exposure neutralization is only possible only 8-24hr later. Thus, a protective recall response would still need to be sufficiently rapid to provide sterilizing immunity. Regardless, since protection can be observed via passive transfer of HPV L1-VLP or L2 antisera in the murine, canine and rabbit challenge models [281, 294, 306], this suggests that such a recall response is not required.

Our study shows for the first time that vaccination with a HPV L2 immunogen in human patients can elicit an immune response that is sufficient to protect naïve animals against vaginal infection. This protection was shown by performing passive transfer studies using sera from individual human patients vaccinated with the fusion protein made from HPV16 L2E6E7 (TA-CIN) (Table 6 and Figure 17). The responses detected to this L2 vaccine were weak suggesting the need for an adjuvant. However a subset of these patient sera that showed a neutralizing response conferred robust protection against a large inoculum of purified virus instilled in the genital tract of the naïve mouse, even at ~100-fold dilution of the patient sera. Taken together, our observations argue that surprisingly low titers of neutralizing antibodies are sufficient for protection, and is consistent with prior pre-clinical studies using animal antisera or animal neutralizing monoclonal antibodies to L2 or L1 VLP [281, 300, 307].

Since neutralizing antibodies are the relevant immune correlate and low titers are sufficient for protection, the development of sensitive, robust and high throughput assays for HPV neutralizing antibodies regardless of immunogen is important for clinical development of second generation HPV vaccines, especially those based on L2. As mentioned earlier, although the L1-PBNA developed by Pastrana and colleagues is very sensitive for detection of vaccine type L1-specific neutralizing antibodies, the detection of L2-specific neutralizing antibodies and L1-specific cross-neutralizing antibodies has been problematic. As a result of

these limitations, several *in vitro* assays including the HT-PBNA[295], the FC-PBNA[13] and the L2-PBNA [11] have been developed to improve the analytic sensitivity for detection of both L1 and L2 neutralizing antibodies over the original L1-PBNA approach. While these assays show clear improvements in sensitivity particularly towards detecting L2-specific neutralizing antibodies in mouse or rabbit sera, further validation with human sera vaccinated with either HPV L1 or L2 immunogens is required. This in turn has been particularly challenging for L2-specific neutralizing antibody detection since natural responses to L2 are rare, and clinical testing of HPV L2-specific vaccines has been limited.

In this study, we attempted to validate our previously developed FC-PBNA's using human patient serum. To do so, we compared its performance to the L1-PBNA. We first compared sensitivity in detecting both L1-VLP specific and cross-neutralizing titers. With respect to the former, both assays were similarly sensitive for detecting HPV L1-specific neutralizing antibodies from natural infection (using the WHO international standards to HPV16 and 18 antibodies) or after VLP vaccination (Figure 15). Together, the results suggest that furin pre-cleavage of PsV does not compromise the key conformational and type-specific L1 neutralizing epitopes [44, 294].

When we previously tested both assays using a single pooled serum from Cervarix-vaccinated mice, the FC-PBNA was more sensitive than the L1-PBNA in detecting HPV31 and HPV45 L1-cross neutralizing antibodies [13] suggesting that furin pre-cleavage might better reveal sub-dominant cross-neutralizing L1 epitopes [67, 273, 308]. To reassess and validate our previous findings, we tested Gardasil patient sera in both assays against HPV31 and 45. However, only a minor fraction of the patient sera had a detectable neutralizing titer for these types (Table 5). This may reflect the lower titers induced in these patients versus mice and also the higher titers elicited by Cervarix versus Gardasil. Additionally, Gardasil may be less protective against HPV45 than Cervarix. This was based previous clinical observations (reviewed in [278]) and our own whereby we observed our FC-PBNA was able to detect

cross-neutralizing titers in sera pooled from ten mice vaccinated three times with Cervarix vaccinated mice against HPV31 and 45 but no neutralizing titers (<50) was detected in either assay for these types in a similarly pooled serum from ten mice vaccinated three times with Gardasil (data not shown). Given the demonstrated ability of Gardasil to protect patients and mice from HPV31 [8, 309], this suggests that there is still need to improve the sensitivity of the neutralization assays for detecting L1 cross-neutralizing antibody titers. The application of a more sensitive reporter may be helpful in this regard, as suggested by the use of Gaussia luciferase in the HT-PBNA and its greater sensitivity compared to the L1-PBNA. This is further supported by the report that the HT-PBNA is more sensitive compared to the SEAP-based L1-PBNA and our own findings that the SEAP-based L1-PBNA is highly correlative and similarly sensitive as the FC-PBNA using firefly luciferase as the reporter (Figure 15C). Further, the FC-PBNA could potentially be adapted for use with robotics as described for the HT-PBNA to improve sensitivity, reproducibility and throughput.

VIN patients vaccinated with TA-CIN produced weak HPV16 L2-specific serum antibody responses detectable by ELISA (Figure 16). However, ELISA does not discriminate between neutralizing and non-neutralizing L2 antibodies. Indeed, the neutralizing epitopes compromise a very small proportion of the entire L2 sequence, and it appears that non-neutralizing epitopes toward the the C-terminus of L2 can become immunodominant over neutralizing epitopes at the N-terminus. Hence, the results show that the exclusion of non-neutralizing epitopes on an immunogen like L2 must be carefully considered in HPV L2 design. In addition, although the L2 ELISA is sensitive in detecting overall L2 antibodies, an *in vitro* neutralization assay is of critical importance to evaluate the true neutralization efficacy of candidate L2 vaccines. The weak and inconsistent L2-specific responses to 125µg TA-CIN here, were also in line with a previous observation in a different cohort of VIN patients vaccinated with a higher dose of TA-CIN (533µg) which exhibited weaker responses compared to that of healthy volunteers [296]. Additionally, vaccination of mice with TA-CIN alone also elicits similarly weak L2-specific neutralizing antibodies, but upon use of an

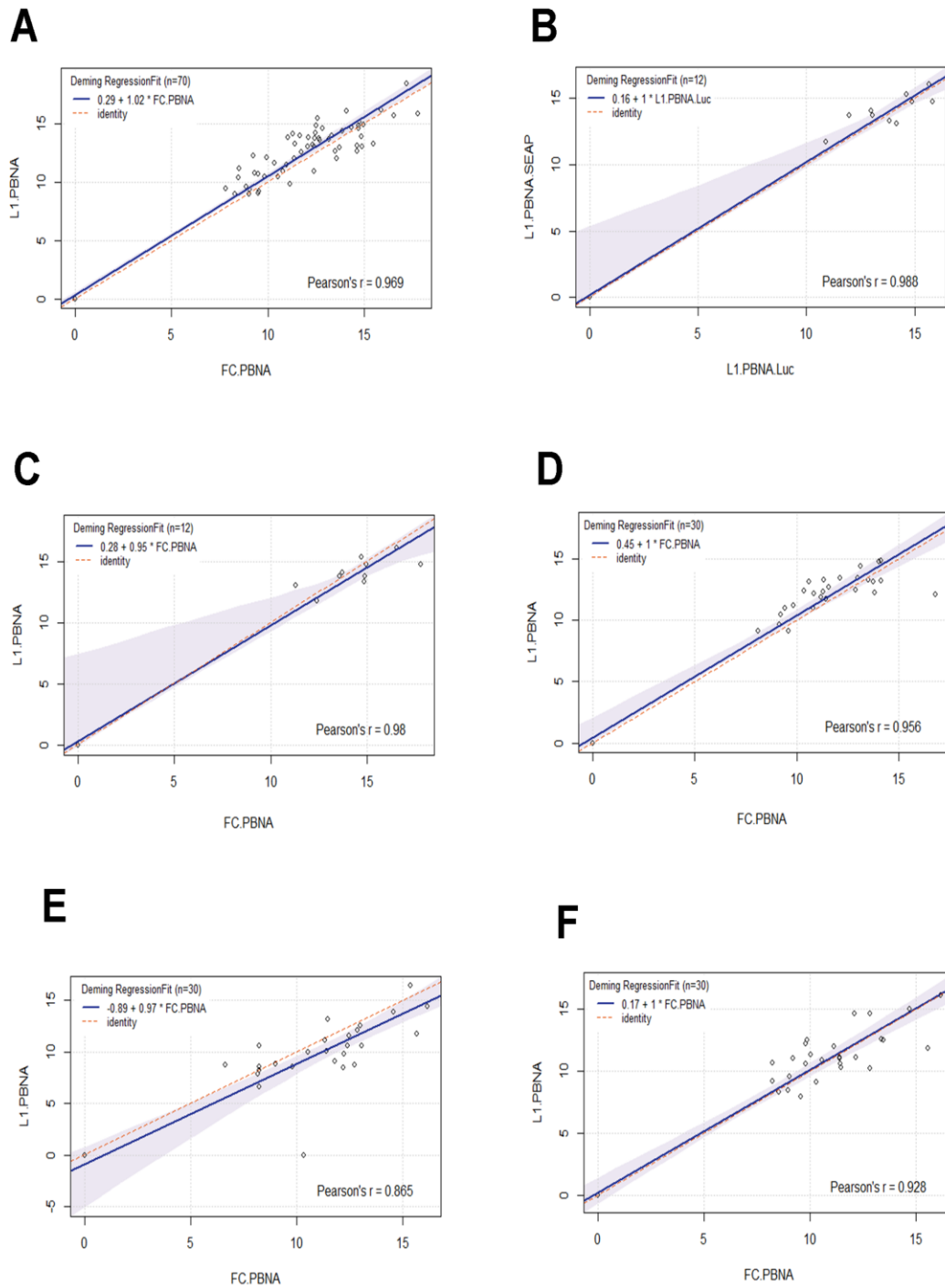
adjuvant with TA-CIN, for example the saponin GPI-0100, consistently elicits potent L2-specific neutralizing antibody responses. Notably, these neutralizing antibody titers, although strongly protective, were still lower than those elicited by L1 VLP vaccines [310].

With respect to L2 neutralization, the FC-PBNA was more sensitive compared to the L1-PBNA for detecting the weak L2 neutralizing antibody titers in the sera of patients vaccinated with TA-CIN (Table 6). The FC-PBNA was also more consistent than the L1-PBNA with L2-specific protective titers determined by passive transfer studies in the mouse challenge model. Indeed, the EC50 titers for protection were remarkably similar to the *in vitro* titers for neutralization measured by the FC-PBNA (Table 6 and Figure 17). This observation contrasts a recent study by Longet *et al* wherein the murine model was 200-500-fold more sensitive than *in vitro* assays [300]. However, Longet *et al* utilized the H16.V5 murine monoclonal antibody or mouse anti-L1 VLP antisera [300], whereas our study tested L2-specific human sera. The discrepancy may reflect preferential stability and/or transport of mouse versus human IgG to the challenge site in the mouse vagina. Alternatively, the close co-relation in titers between the FC-PBNA and *in vivo* model, could reflect that the mechanisms of L2-mediated neutralization are similar both *in vitro* and *in vivo* unlike L1-mediated neutralization as described previously by Day and colleagues [294].

We also observed a similar sensitivity for the FC-PBNA and a recently described L2-PBNA assay for L2-specific neutralizing antibodies in human serum (Intercept = 0.22 [95% CI= -0.257,0.70], Slope = 1.33 [95% CI= 1.15,1.51]), Pearson's  $r=0.93$ ) (Figure 17F). Thus the L2-PBNA and FC-PBNA might be used interchangeably as both are simpler and higher throughput than the passive transfer in the murine challenge model established by Roberts and colleagues [18], although the *in vivo* method is the most sensitive at present and potentially a more biologically relevant approach [300]. However, this requires further validation and highlights the need for additional international standard serum sets for L1 and L2-specific neutralizing antibodies to facilitate comparison of assay formats and validation across

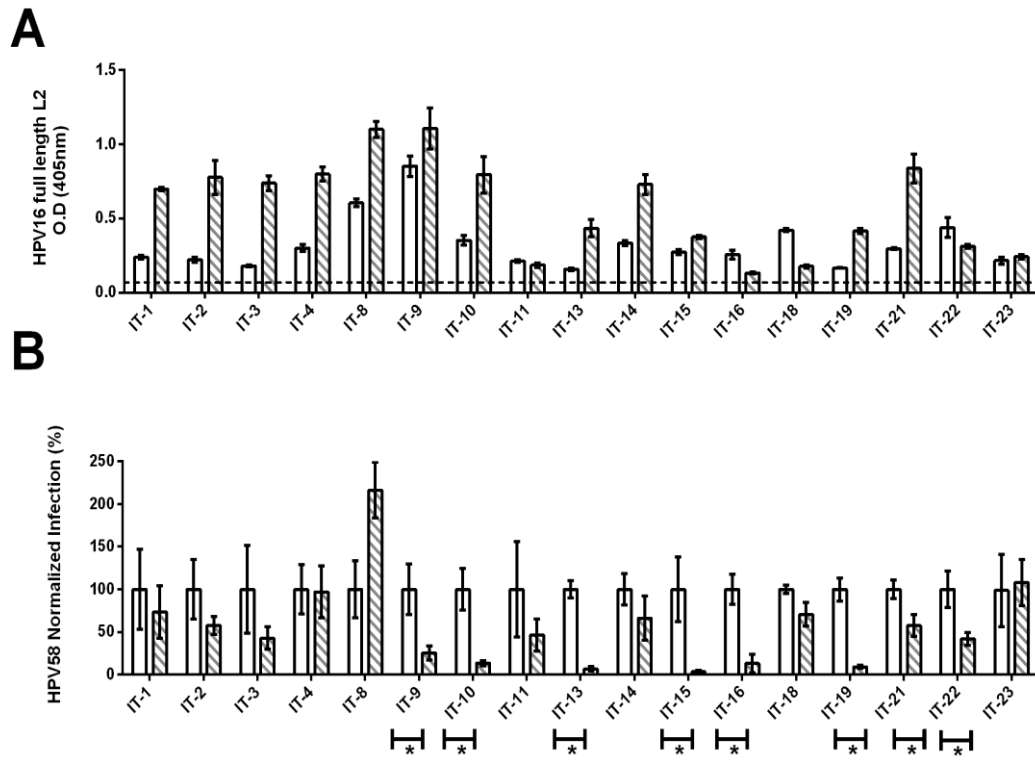


different laboratories performing the same assays. We previously reported that the FC-PBNA was >10-fold more sensitive than the L1-PBNA for detection of L2-specific neutralizing antibodies whereas the L2-PBNA reported 100-10,000-fold [11], the limited number of detectable responses and available samples prevents us from determining the absolute magnitude of this difference using human sera. Nevertheless, these findings indicate that the FC-PBNA, as well as the L2-PBNA developed by Day and colleagues [11], are sensitive assays for L1 VLP or L2-specific neutralizing antibody in human serum, and potentially valuable for monitoring immune responses to prophylactic HPV vaccination.

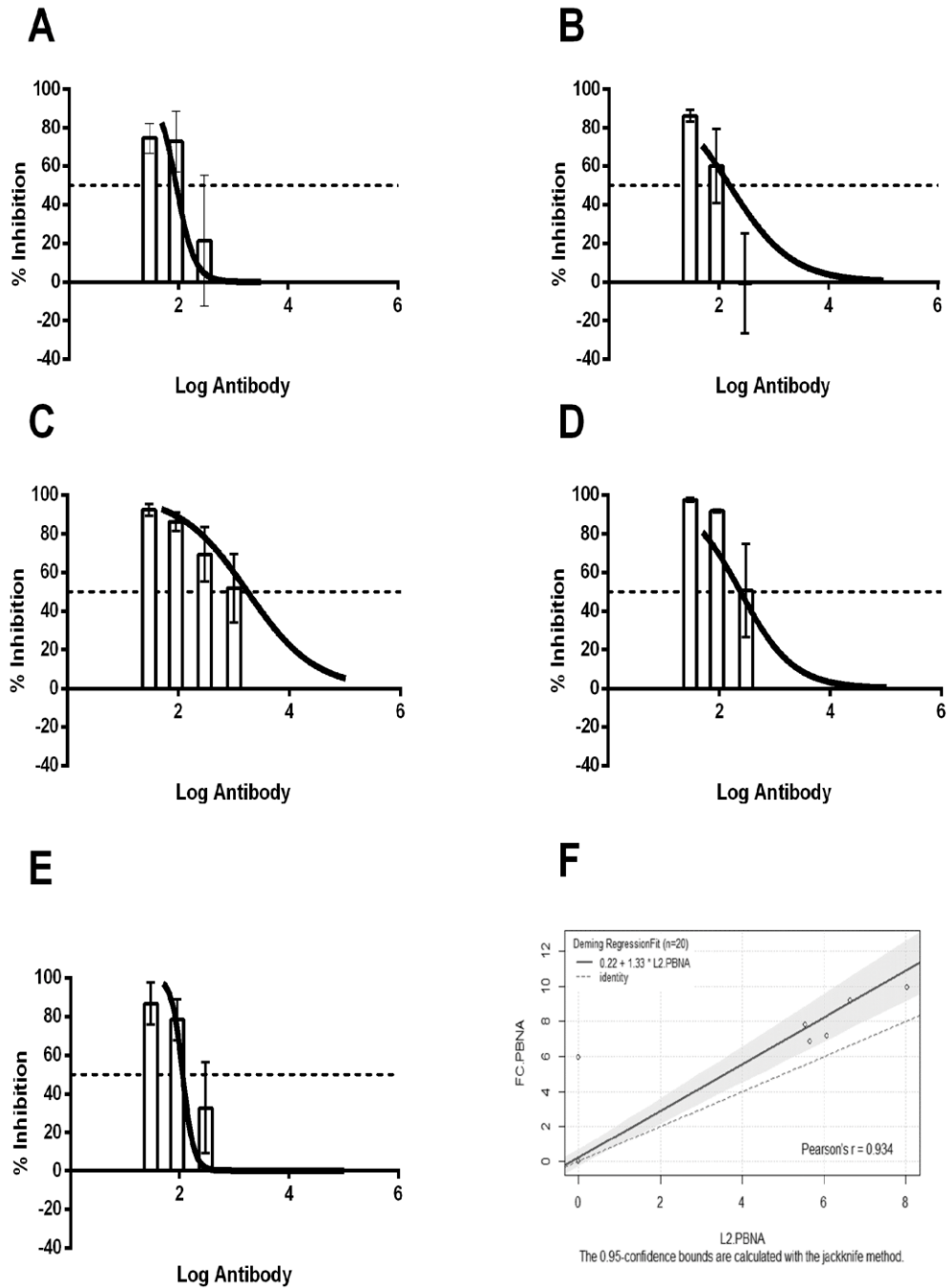


**Figure 15. Correlation of neutralization assays.** The estimated EC50 values of each patient sample from the respective assays were log2 re-transformed and plotted using R package mcr. Values for Person's r, slope and intercepts were rounded to 2 decimal places. The 0.95-confidence bounds are calculated with the bootstrap (quantile) method. Comparison of HPV16 VLP vaccinated patient sera (n=70) in FC-PBNA versus L1-PBNA (A). Comparison

of L1-PBNA (B) and FC-PBNA (C) with previous findings (n=12) by Pastrana and colleagues using SEAP-based L1-PBNA [254]. Comparison of n=30 Gardasil vaccinated patient sera in FC-PBNA and L1-PBNA against HPV16 (D), HPV18 (E) and HPV6 (F).



**Figure 16. Assessment of TA-CIN sera (n=17).** HPV16 full length L2 ELISA using sera of patients vaccinated with TA-CIN was performed in triplicate and is presented as mean  $\pm$  Standard deviation (A). Results of *in vivo* passive transfer studies with patient sera. Asterisk indicates significant difference in mean infection of the five mice per group  $\pm$  standard error against intra vaginal HPV58 challenge observed between pre- and post-vaccinated sera (100 $\mu$ L per mouse) (B). For both figures, white bars indicate pre-vaccinated serum and hashed bars indicate post-vaccinated serum.



**Figure 17. FC-PBNA correlates well with both *in vivo* murine challenge model and L2-PBNA.** Comparison of FC-PBNA EC<sub>50</sub> fitted *in vitro* neutralization titers curve (black line) with passive transfer studies using titrated dilutions (10 $\mu$ L, 33 $\mu$ L, 100 $\mu$ L) of TA-CIN patient sera (white bars) where IT-9 (A), IT-10 (B), IT-13 (C), IT-15 (D), IT-16(E). For IT-13, sera did not cross 50% inhibition at 10 $\mu$ L and thus, the experiment with a further 3 $\mu$ L dilution was repeated to assess *in vivo* inhibition. Comparison of serum titers of patients vaccinated with

TA-CIN as detected by FC-PBNA and the L2-PBNA and plotted using R (see text for Pearson's  $r$ , slope and intercept) (F).

**Table 5. Gardasil patient serum (n=30) EC50 titers determined with L1-PBNA and FC-PBNA for HPV 6, 16, 18, 31 and 45.** Neutralization titer is based on the reciprocal of the dilution that causes 50% reduction in luciferase activity. Titers were rounded off to the nearest whole number. \*Patient 121 was sampled within a week after receiving the third Gardasil dose. \*\*Clinical information on Patient 135 was unavailable.

Patient	Gardasil Dosage	Length of time between last vaccination and blood draw	Mean Neutralization Titers (EC<50)									
			HPV 6		HPV 16		HPV 18		HPV 31		HPV 45	
			FC-PBNA	L1-PBNA	FC-PBNA	L1-PBNA	FC-PBNA	L1-PBNA	FC-PBNA	L1-PBNA	FC-PBNA	L1-PBNA
105	0	N/A	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
119	1	1 months	7352	1210	278	553	300	100	<50	<50	<50	<50
126	1	4 months	11281	6021	11581	10024	2576	2180	<50	<50	<50	<50
104	1	6 months	<50	<50	<50	<50	<50	<50	<50	<50	<50	<50
128	1	2 years	<50	<50	<50	<50	<50	<50	109	<50	85	<50
124	2	2 months	4381	26098	2543	9943	8183	5995	407	572	<50	<50
112	2	1 year	758	249	4457	10826	289	228	<50	<50	<50	<50
130	2	1 year	1054	2635	765	542	897	376	<50	<50	<50	<50
131	2	1 year	26460	33253	<50	<50	<50	<50	<50	<50	<50	<50
121	3	Immediate*	2768	1574	596	1384	5752	2955	<50	<50	<50	303
109	3	2 mths	1514	1934	1536	8955	2701	1055	<50	<50	<50	<50

114	3	4 months	900	1542	14342	4746	52910	3358	54	79	<50	<50
108	3	1 year	375	322	2522	5102	6849	431	2880	786	<50	<50
111	3	1 year	497	358	1304	5416	3570	536	<50	<50	<50	<50
120	3	1 year	1264	557	3005	6489	4884	900	<50	<50	<50	<50
122	3	1 year	7372	25482	687	1991	1481	986	139	133	<50	<50
129	3	1 year	900	4851	926	2349	300	372	<50	<50	<50	<50
134	3	1 year	2236	4041	2349	3688	2859	9102	102	105	<50	<50
115	3	2 years	2700	2221	13848	8948	8685	1534	<50	<50	<50	1545
116	3	2 years	2856	1259	7528	5627	300	300	<50	<50	<50	<50
118	3	2 years	2700	2099	2740	3294	42235	88975	<50	<50	8084	<50
123	3	2 years	933	5885	17911	28851	7579	4367	<50	192	<50	<50
125	3	2 years	589	2095	566	800	100	416	<50	<50	<50	<50
127	3	2 years	72900	72900	9035	21040	521	466	<50	214	<50	<50
132	3	2 years	527	760	1831	4597	300	1522	<50	<50	<50	1120
133	3	2 years	47870	3719	17849	9236	73676	21269	398	261	7114	2748
113	3	4 years	4601	2208	8022	10883	5408	1517	<50	<50	<50	<50
117	3	4 years	300	603	112791	4431	1303	<50	<50	<50	<50	<50
107	3	5 years	300	1610	1773	2005	4702	364	<50	<50	<50	<50
135**	N/A	N/A	10627	6248	16560	28160	24690	14689	145	149	1657	357



**Table 6.** Assessment of sera titers of patients vaccinated with TA-CIN using the L1-PBNA, FC-PBNA and titrated passive transfer assay. Titers were rounded off to the nearest whole number. ND= Not done. ND\*= Not done due to limited sera available

TA-CIN patient no.	Post-Vaccination Sera titer		
	L1-PBNA titer [95% Confidence Interval]	FC-PBNA titer [95% Confidence Interval]	Titrated <i>In Vivo</i> Assay titer [95% Confidence Interval]
1	<50 [N/A]	<50 [N/A]	N.D
2	<50 [N/A]	<50 [N/A]	N.D
3	<50 [N/A]	<50 [N/A]	N.D
4	<50 [N/A]	<50 [N/A]	<50 [N/A]
8	<50 [N/A]	<50 [N/A]	<50 [N/A]
9	130 [23 to 736]	113 [13 to 995]	135 [40 to 460]
10	94 [20 to 442]	147 [28 to 775]	101 [50 to 203]
11	<50 [N/A]	<50 [N/A]	N.D
13	<50 [N/A]	1000 [517 to 1934]	923 [350 to 2436]
14	<50 [N/A]	<50 [N/A]	N.D
15	<50 [N/A]	229 [115 to 453]	305 [139 to 671]
16	<50 [N/A]	115 [40 to 326]	191 [88 to 415]
18	<50 [N/A]	<50 [N/A]	N.D
19	<50 [N/A]	512 [161 to 1626]	N.D*
21	<50 [N/A]	<50 [N/A]	N.D*
22	<50 [N/A]	<50 [N/A]	N.D*
23	<50 [N/A]	<50 [N/A]	N.D

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### **III. Sero-epidemiology of HPV16 L2 and generation of human chimeric papillomavirus L2-specific antibodies**

#### **ABSTRACT**

The World Health Organization (WHO) has developed L1 VLP-specific reference sera pooled from HPV16 or HPV18-infected patients. Presently, no such standard exists for L2-specific neutralizing antibodies despite several HPV L2-based vaccines are nearing/beginning clinical development. To address the sero-prevalence of HPV16 L2 antibodies and potentially locate a suitable pool of HPV16 L2-positives for a standard, we first screened for L2-reactivity a total of 1078 sera obtained from four prior clinical studies: a population-based (n=880) surveillance study with 10.8% high risk HPV DNA prevalence, a cohort study of women (n=160) with high-grade cervical intraepithelial neoplasia (CIN), and two phase II trials in women with high-grade vulvar intraepithelial neoplasia (VIN) testing imiquimod therapy combined with either photodynamic therapy (PDT, n=19) or vaccination with a fusion protein comprising HPV16 L2, E7, and E6 (TA-CIN, n=19). Sera were screened sequentially by HPV16 L2 ELISA, then Western blot. Seven of the 1078 sera tested had spontaneous L2-specific antibody, but it was not detectably neutralizing for HPV16. As an alternative, we substituted human IgG1 sequences into conserved regions of two rodent monoclonal antibodies (mAb) specific for L2 neutralizing epitopes at residues 17-36 and 58-64 of HPV16 L2, creating JWW-1 and JWW-2 respectively. These chimeric mAbs retained neutralizing activity and together reacted with 33/34 clinically-relevant HPV types tested. In conclusion, our inability to identify an HPV16 L2-specific neutralizing antibody response even in sera of patients with active genital HPV disease suggests sub-dominance of L2 protective epitopes and the value of the chimeric mAbs JWW-1 and JWW-2 as standards for immunoassays to measure L2-specific antibodies in patients.

## INTRODUCTION

Persistent infection with a high risk human papillomavirus (hrHPV) is a necessary, although insufficient cause of cervical cancer and subsets of other anogenital and oral-pharyngeal cancers [2, 139]. Despite the licensure of two HPV vaccines based on L1 virus-like particles (VLP) that have been shown to be highly effective, cervical cancer remains as the third most common cancer worldwide, with 80% of cases occurring in the developing world [311]. This disparity reflects both limited vaccine implementation and certain technical and logistic issues in cervical cancer screening in the developing world.

The licensed L1 VLP vaccines (Gardasil®, Merck & Co. and Cervarix®, GSK) target the two most problematic hrHPV genotypes, HPV16 and HPV18, which together cause ~70% of all cervical cancer cases. Gardasil® also contains L1 VLP types derived from HPV6 and HPV11, and provides protection from benign genital warts caused by these viruses [284]. However, neither licensed vaccine targets either the remaining oncogenic HPV genotypes responsible for 30% of all cervical cancers [312], or the cutaneous HPV genotypes associated with non-melanoma skin cancer, most commonly in persons with epidermodysplasia verruciformis (EV) or who are immunocompromised. Emerging data also suggest that the genotype distribution of HPV that cause cervical pathology differ in different countries and even ethnic origins [313-315]. Although some cross-protection against very closely related HPV types has been identified in the current vaccines, it does not cover all hrHPV and its longevity is uncertain [176, 278].

To increase the breadth of coverage, a vaccine containing VLPs from seven oncogenic HPV types (as well as HPV 6 and 11) has been developed and approved by the FDA with the intent of achieving >90% protection from cervical cancer [5]. However increasing the valency is likely to increase manufacturing costs, therefore discouraging uptake where vaccination is most needed [316] as >85% of the cervical cancer disease burden lies in low resource settings, reflecting in

part insufficient resources for screening programs and HPV vaccination [317]. Thus, there remains a clear need to develop an affordable vaccine that broadly protects against all hrHPV types.

Vaccination with the minor capsid protein L2 has potential as an approach to comprehensive and inexpensive HPV vaccination as the N-terminal protective epitope sequences are well conserved. Preclinical vaccine studies demonstrate that this region of L2 can elicit protection against diverse papillomavirus types [68, 120, 126]. Further, the passive transfer of L2-specific neutralizing antibodies into naïve animals is sufficient for protection from experimental challenge, providing evidence to support their central role in protective immunity. Additionally, because the neutralizing epitopes of L2 are linear and conserved, in contrast to the type-restricted conformational epitopes of L1-VLP vaccines, cross-reactive L2 vaccines can be produced as a single antigen in bacteria, which may reduce manufacturing costs compared to the licensed L1 VLP vaccines. However, while L2-specific antibodies are broadly reactive, their ability to neutralize less evolutionarily-related types is weaker than for the cognate type. Therefore, we and others have sought to enhance cross-protection by either the concatenation of L2 epitopes derived from several HPV types or displaying L2 epitopes on VLPs. Both approaches can broadly protect vaccinated animals against experimental challenge with diverse HPV types [7, 8, 10, 123, 318].

To lay the groundwork for L2 vaccine trials, the prevalence of L2-specific neutralizing antibodies should be understood in both healthy patients and those with HPV disease. Pre-clinical studies suggest that L2 is immunologically subdominant to L1 in the context of the capsid suggesting that responses to infection with genital HPV are likely to be rare and/or weak when they are detectable. To date, studies addressing L2-specific antibody responses to HPV infection are both limited in number and inconsistent with respect to sero-prevalence [319-325]. Some of these inconsistencies may reflect reliance upon a single assay format and/or utilizing only bacterially-

expressed L2 antigens [319-325]. To address this, here we examined the serologic responses to HPV16 L2 in both an unscreened healthy population at risk for HPV infection and in patients with high grade cervical or vulvar intraepithelial neoplasia (CIN2/3 or VIN2/3 respectively), using HPV16 L2 ELISA followed by a confirmatory Western blot analysis (i.e. a two-step approach). We also examine whether topical application of a Toll-like receptor 7 (TLR7) agonist, imiquimod, at the lesion site and either ablation of the lesion with photodynamic therapy or intramuscular vaccination with HPV16 L2E7E6 fusion protein (TA-CIN) elicit serum antibody responses to L2.

In addition to sero-prevalence, validated serologic assessment methods that are both functionally sensitive as well as high-throughput will be required to assess neutralizing antibody responses of patients. Recently, several new *in vitro* assays with enhanced sensitivity to L2-specific neutralizing antibodies have been developed [11, 13, 295]. A key requirement for their routine validation is a reproducible positive control [326]. Indeed, the need for standardization of assays for assessing responses to HPV L1-VLP vaccination led the World Health Organization (WHO) to develop international serologic standards for HPV16 and HPV18 L1-VLP reactive antibodies respectively, using serum pooled from HPV-infected women [326, 327]. Here, we sought to follow a similar approach in generating a standard for future L2-based vaccination validation studies, by identifying patient sera with L2-reactive neutralizing antibody.

## **MATERIALS AND METHODS**

### **Human samples**

The studies herein using human sera were reviewed and approved by the Johns Hopkins University Internal Review Board. Human sera were collected previously from women in four published clinical cohorts: 1) a 38% random sample (n=880) of sera collected as part of a population-based (n=2331) surveillance study in India with 10% high risk HPV DNA prevalence

(*Community Access to Cervical Health (CATCH) Study*) [328] (see Tables 7 and 8 for patient demographics), 2) Patients (n=160) with high grade cervical intraepithelial neoplasia (CIN) [205] (see Table 7 and 9 for patient demographics), 3) Patients (n=19) enrolled in a Phase II study with high-grade vulvar intraepithelial neoplasia (VIN) who were treated with imiquimod for 8 weeks followed by photodynamic therapy [329], and 4) high-grade VIN patients (n=19) who were treated with imiquimod for 8 weeks, followed by vaccination three times at monthly intervals with a fusion protein comprising HPV16 L2, E6, and E7 (TA-CIN) [296]. On both phase II trials studies, sera were obtained prior to and after imiquimod treatment (weeks 0 and 10 respectively) and after PDT/TA-CIN vaccination (week 26).

**Enzyme-linked immunosorbent assays (ELISA) and neutralization assays** Immobilon plates (Nunc) were coated with 500ng/well of purified baculovirus-derived HPV-16/18 L1-VLPs [330], HPV-16/31/45/58 pseudovirions (PsVs), or bacterially-expressed and 6His-tagged HPV16 L2 [198], or L2 $\alpha$ (11-88)x5 multimer polypeptide [8]. The plates were incubated overnight at 4°C. Wells were then blocked with 1% bovine serum albumin (BSA)–PBS for 1h at 37°C, and incubated with human sera at 1:50 dilution for 1h at 37°C. Following a wash step with PBS-0.01% (v/v) Tween 20, peroxidase-labeled Sheep anti-human (GE Healthcare) diluted 1:5000 in 1% BSA–PBS was incubated for 1h. The plates were then washed and developed with 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)s (Roche) for 10 min, and the absorbance was measured at 405 nm. For JWW-1 and WW-1 ELISA reactivity comparisons to HPV16 L2 and L2 $\alpha$ (11-88)x5, a total of 400ng of antibody in 100 $\mu$ L/well (26.7nM) was utilized as the starting concentration. Neutralization assay comparison between JWW-1, JWW-2 and control IgG was done using our recently described furin-cleaved pseudovirion-based neutralization assay (FC-PBNA) [331].

### **SDS-PAGE and Western blotting**

4-15% gradient SDS-PAGE gels were loaded with 40µg of lysate of 293TT cells transfected with expression vectors for either eGFP or HPV16 L2. The gels were run at 110V for 1.5 to 2 hours. The proteins were then transferred onto a nitrocellulose membrane for 90 min at 50V. The membranes were blocked for one hour at RT with 5% non-fat dried milk in phosphate-buffered saline containing 0.1% (v/v) Tween 20 (PBST). The membranes were then incubated with sera diluted at 1:300 or higher (highest 1:5000 due to limiting sera) in 5% milk in PBST overnight at 4°C. The membranes were then washed for 10 min three times with PBST. Membranes were incubated with anti-human IgG HRP secondary diluted 1:5000-10,000 in 5% milk in PBST and incubated for 1h at RT. Membranes were then washed three times with PBST for 10 min each time. Chemiluminescence substrate was added to develop the membranes.

### **Design and generation of JWW-1 and JWW-2 expression plasmid**

The rat monoclonal antibody WW-1 is broadly neutralizing and reactive with HPV16 L2 17-36 [13]. The WW-1 heavy and light chain sequences were obtained by sequencing the hybridoma cDNA (Aldevron, Fargo, USA). Variable region sequences for the mouse monoclonal antibody MAb24B were derived from [132]. Both the constant light and heavy chain regions of WW1 and MAb24B were replaced with the constant light and heavy chain regions of human IgG1 sequence and directly synthesized (Biobasic Inc, Ontario, Canada). The chimeric heavy and light chains of WW-1 and MAb24B were each cloned into a double expression vector, pVITRO1-neo-mcs (Invivogen, San Diego CA) to generate the JWW-1 and JWW-2 plasmids respectively. Plasmids to produce these monoclonal antibodies can be readily obtained from [https://www.addgene.org/Richard\\_Roden/](https://www.addgene.org/Richard_Roden/)



### **Expression and Purification of JWW-1 and JWW-2**

For initial testing,  $5 \times 10^6$  293TT cells were seeded into a 6 well plate the day before transfection. The cells were transfected with either pVITRO1-neo-mcs empty vector template (mock transfection) or JWW-1/JWW-2 using Mirus TransIT 2020 (Mirus Bio, Madison WI) according to the manufacturer's protocol and maintained in Opti-MEM reduced serum media (Gibco, Life technologies, Grand Island NY). After 72 hours, the supernatant was clarified by centrifugation (1600rpm, 4.5 minutes at room temperature) and passage through a 0.2 $\mu$ m filter (Milipore, Billerica, MA) to remove cellular debris. The filtrate was used for Western blot studies at a 1:100 dilution to determine reactivity. For large scale purification, the cell line Expi293<sup>TM</sup> was utilized as per the manufacturer's (Life technologies, Grand Island NY) instructions and purified using HiTrap Protein G HP columns (GE healthcare BioSciences, Pittsburgh, PA). The quantity of antibody was determined using a Pierce<sup>TM</sup> BCA Protein Assay kit, per the manufacturer's (Pierce) instructions. Purity was assessed by SDS-PAGE and Commassie blue staining.

### **Statistics and data analysis**

For all ELISAs, the mean absorbance and standard deviation (s.d.) of the entire study cohort was first calculated. Subsequently, a positive ELISA value for HPVL2 was designated as an O.D value  $> \text{mean} + 3 \text{ s.d.}$  Results of ELISA screens were plotted using R package ggplot2. FC-PBNA neutralization titer was defined as the reciprocal of the dilution that caused 50% reduction in luciferase activity. FC-PBNA comparisons of RG-1, JWW-1 and JWW-2 were done in triplicate and titrated 2-fold with a starting concentration of 200nM. The values were plotted on Graphpad Prism 6 using the non-linear model  $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{LogEC50} - X) * \text{HillSlope}))}$ .

## RESULTS

### **Seroprevalence of HPV16 L2-specific antibody in serum collected an unscreened population at high risk for HPV**

Since the WHO reference reagents for human PV L1-antibodies to HPV16 and HPV18 are based on serum pooled from several infected patients, we attempted to screen for L2-reactive sera from patients who are at high-risk for or have active HPV genital disease. We first assessed the prevalence of HPV16 L2-reactive serum antibody responses in a random subset of women enrolled in the 'CATCH' (*Community Access to Cervical Health*) study (Table 7) designed to evaluate the feasibility and impact of introducing cervical screening methods in a previously unscreened population in Medchal Mandal, Andhra Pradesh, India [328]. Using ELISA assays, sera from 880 women enrolled in the CATCH study were screened for reactivity with full length HPV16 L2 protein. When employing a cut-off of mean O.D. + 3 s.d. by our L2 ELISA, eighteen of the 880 sera screened were considered potentially positive (Table 8, Patient 1-18). Since the recombinant HPV16 L2 antigen used in the ELISA was purified from *E. coli*, there was a possibility that bacterial protein-specific antibody responses might contribute to false positives by reacting with bacterial contaminants in the HPV16 L2 antigen. Therefore, the 18 HPV16 L2 ELISA putative positive sera were re-screened in a Western blot-based assay, in which lysates of 293TT cells transfected with either an expression vector for HPV16 L2, or GFP as a negative control, were probed with individual patient sera and a peroxidase-linked secondary antibody specific to human IgG. While the presence of the ~70kDa HPV16 L2 protein in the 293TT lysates was confirmed by Western blot using the mouse monoclonal antibody RG-1, only 6/18 putative positive sera reacted specifically with a band of ~70kDa in the lysates of 293TT cells expressing HPV16 L2 (summarized in Table 8). This finding suggests that despite a cervical high risk HPV DNA prevalence of 10.8% (as determined by Qiagen hc2 assays) in the CATCH study patients (Table 7), only a minor (0.68%) fraction of their sera was reactive with HPV16 L2 (by both ELISA and Western blot).

HPV-infected individuals frequently mount a type-specific L1 VLP-reactive serum antibody response [326]. Therefore the sera of these 18 patients from the CATCH study identified in the HPV16 L2 ELISA screen were also tested for reactivity in two ELISA assays using either baculovirus-derived HPV16 or 18 L1-VLP and previously defined cut-off and standards [330]. Importantly, only 1/6 of the verified HPV16 L2-reactive sera was also reactive in the HPV16 L1-VLP ELISA. None of the 6 patients also displayed reactivity against HPV18 L1-VLP (Table 8).

Given that L2 is highly conserved, it is possible that the negative HPV16 and HPV18 L1-VLP ELISA results were due to infection by other related HPV types. To explore this possibility, we performed HPV-31/45/58 pseudovirion ELISAs with the patient sera. However, none of these 6 HPV16 L2 reactive sera (determined by both ELISA and Western blot) of the CATCH study patients reacted with these additional HPV genotypes either.

### **HPV16 L2 seroreactivity of women with HPV16+ CIN**

We next assessed for HPV16 L2-reactive serum antibodies a cohort of patients at the Johns Hopkins Hospital (n=160, mean age 29.5 years) with cervical intraepithelial neoplasia 2/3 (CIN2/3) (Table 9) enrolled in a longitudinal cohort protocol, in which serially collected ( $\leq 4$ ) blood samples were obtained. A total of 298 serum samples from this cohort were available for testing. Although the cervical swabs of 99 of the 160 patients were HPV16+ (62%), only one of their sera (Table 8, sample 19) was reactive to HPV16 L2 by both ELISA and Western blot assays (Figure 18B). The serum was provided by a patient diagnosed with an HPV16+ CIN2 at study entry. Her cone excision at study week 16 was benign suggesting that patient's lesion underwent spontaneous regression. A year later she was diagnosed with an HPV16-negative CIN3, which also regressed. She had donated serum samples at four visits over this period and each was similarly reactive to HPV16 L2 both via ELISA and Western blot (Table 8, sample 19A-D), but none to either HPV16 or HPV18 L1-VLP by ELISA (Table 2). Together, these data

suggest that a systemic antibody response to HPV16 L2 was uncommon even in women with HPV16+ CIN2/3.

### **Neither imiquimod nor PDT elicit an HPV16 L2 antibody response in VIN patients**

The low prevalence of a serum antibody response to HPV16 L2 detectable in the cohort of women with HPV16+ CIN2/3 may reflect HPV infection that is confined to mucosal epithelium. To address the possibility that infection at a different site such as the vulva which might be more immunogenic, sera of 19 patients with high grade vulvar intraepithelial neoplasia 2/3 (VIN2/3), of which 15 were HPV16+, obtained during a phase II study were tested for HPV16 L2-specific antibody [329]. None were positive (Figure 18C, 1D).

The lack of an L2 antibody response might also reflect the localized and non-lytic replication of HPV and thus minimal inflammation associated with genital HPV16 infection. Therefore we reasoned that topical application of the TLR7 agonist imiquimod at the lesion site might act as an adjuvant that would promote a serologic response to L2. The patients were treated subsequently with imiquimod applied to the lesion once in the first week, twice in the second week, and three times weekly for six subsequent weeks. At week 10, one week after completing the imiquimod regimen, a peripheral blood sample was obtained. No L2-specific serologic response was detected in any of the subjects. Following imiquimod treatment, the patients received photodynamic therapy (using methylaminolevulinate cream and red light via an Aktelite 128 (Photocure ASA) set to deliver a dose of 50 J/cm<sup>2</sup>) at weeks 12 and 16 of the study [329]. Peripheral blood was obtained at week 26. No significant differences in L2 ELISA reactivity were identified in within-subject comparisons before versus after treatment (Figure 18C and Figure 1D). This finding suggests that any potential adjuvant effect caused by local innate stimulation with imiquimod, even upon lesion damage with photodynamic therapy was insufficient to elicit detectable systemic L2 responses in the clinical setting of persistent HPV16+ VIN2/3.

### **Vaccination elicits an HPV16 L2 antibody response in VIN patients**

The inability to detect a serologic response against HPV16 L2 in patients with HPV16+ high grade anogenital intraepithelial neoplasia (AGIN) may reflect some technical issue with the assay or that the AGIN patients have a genetic background that is incompatible with the induction of an L2 antibody response. To further investigate, HPV16 L2-specific serologic responses in high grade VIN patients (n=19) treated with imiquimod for 10 weeks followed by three vaccinations with 125µg of a fusion protein comprised of HPV16 L2, E7, and E6 (TA-CIN) were assessed. Consistent with an earlier VIN cohort study, no HPV L2 responses were induced following topical imiquimod application (Figure 18C). However, after vaccination with TA-CIN, 63% of the patient group (12/19) developed an HPV16 L2 response (Figure 18D) which was also detectable by Western blot (Data not shown). Thus the failure to detect an L2 antibody response in HPV16+ VIN patients does not reflect either a technical issue with the assay or the inability of most VIN patients to mount an L2 antibody response.

### **L2 antibody responses induced by HPV infection are not detectably neutralizing**

Our screening efforts yielded 7 patients (see Table 8) with HPV16 L2 antibody verified by both ELISA and Western blot. As 6/7 were also HPV16 L1-VLP ELISA negative, we next assessed if their L2 responses had neutralizing potential. Using the standard *in vitro* HPV neutralization assay against HPV16 pseudovirions, none were detectably neutralizing (all  $EC_{50} < 1:50$ ). It was surprising that the sample 9 was not detectably neutralizing, but this may reflect a greater sensitivity of the HPV16 L1 VLP ELISA. Key neutralizing epitopes of HPV16 L2 have previously been mapped to its first 88 residues [332]. However none of the full length HPV16 L2-reactive sera reacted in an ELISA with the L2 $\alpha$ (11-88)x5, an antigen comprised of amino acids 11-88 from the L2 of HPV-6/16/18/31/39 [8]. This negative ELISA result was consistent with the inability of these sera to neutralize HPV16 pseudovirions (Table 8) despite their reactivity towards full length HPV16 L2 by ELISA and Western blot. Taken together, these

findings suggest that the HPV16 L2 antibody responses identified by ELISA are directed to non-neutralizing epitopes outside of the 11-88 region.

### **Development of chimeric human monoclonal antibodies JWW-1 and JWW-2 as HPV L2 serological standards**

The inability to identify any naturally occurring neutralizing L2 sera subsequently led us to graft two rodent monoclonal antibodies, each targeting a different L2 neutralizing epitope, onto a human IgG1 backbone which results in a chimeric human monoclonal antibody. Chimeric human monoclonal antibodies to L2 represent an alternate approach to develop a serological standard, and also have the additional benefit of reproducibility, ready quantification and limitless supply. HPV16 L2 contains two conserved neutralizing epitopes between 17-36 [68] and 58-81 [132] that are recognized by two broadly neutralizing monoclonal antibodies, WW-1 [13] and MAb24B respectively [132]. WW-1 is a rat monoclonal IgG2a antibody that binds and neutralizes to many more HPV genotypes than the first generation mouse monoclonal antibody RG-1[13]. The mouse monoclonal antibody MAb24B developed by Nakao *et al* is broadly neutralizing against diverse genital HPV types and recognizes HPV16 L2 residues 58-64 [132]. To construct these chimeric monoclonals, these antibody sequences were obtained and chimeric antibodies were constructed for each whereby the variable regions were retained while constant regions of the parental rodent antibodies in both their heavy and light chains were replaced with the equivalent constant regions of a human IgG1. The resultant chimeric sequences encoding both chains derived from WW-1 or MAb24B then were sub-cloned separately into a double expression vector and renamed JWW-1 and JWW-2 respectively.

The chimeric antibodies were expressed in serum-free cultures of human 293 cells after transfection with JWW-1 or JWW-2 and purified from the medium using protein G-coupled beads. To test whether JWW-1 and JWW-2 chimeric antibodies both retained reactivity with L2

and were recognized by a peroxidase-linked secondary antibody to human IgG alongside, each, along with their respective parental rodent monoclonal antibodies, were tested in an ELISA for reactivity to L2  $\alpha$ 11-88x5, an antigen which contains both of their neutralizing epitopes. In addition, the WHO standard serum for HPV16 (05/134), which reacts with HPV16 L1 VLP, as well as the single serum from the HPV16+ CIN2 patient which recognized HPV16 L2 (sample 19, Table 8) outside of residues 11-88, were also tested as controls. Both JWW-1 and JWW-2 bound to L2  $\alpha$ 11-88x5 and this was reflected by the strong recognition via a peroxidase-linked secondary antibody to human IgG in this ELISA assay. However, neither the HPV16+ CIN2 patient serum (sample 19, Table 8) nor WHO standard serum 05/134 reacted in this ELISA (Figure 19A-B) suggesting non-L2 reactivity for the WHO standard and the recognition of L2 epitopes outside the 11-88 region for sample 19. The chimeric antibodies and patient sera were each subsequently tested for reactivity by Western blot with 293TT cells transfected with either full length HPV16 L2 or GFP expression vectors. As expected, JWW-1, JWW-2 and the HPV16+ CIN2 patient serum (sample 19, Table 8) reacted with a ~70kDa band consistent with HPV16 L2 when probed with a peroxidase-linked secondary antibody specific for human IgG, whereas 05/134 was not reactive (data not shown).

The breadth of JWW-1 and JWW-2 reactivity towards pseudovirion preparations of 34 clinically relevant HPV genotypes was next tested via Western blot (Figure 20, and summarized in Table 10). As a control, the presence of the L2 in the pseudovirion preparations was confirmed by re-probing the stripped blots with a broadly reactive rabbit antiserum raised against L2 $\alpha$ 11-88x5 (Figure 20). JWW-1 was able to react with L2 of 29/34 HPV types tested, and was consistent with its parental rat monoclonal antibody WW-1 in failing to recognize only HPV6, 11, 33, 38 and 23 L2. In contrast, JWW-2 was able to bind to 25/34 types, and its spectrum of reactivity was distinct from JWW-1; for example JWW-2 failed to bind to HPV26 and 51 whereas JWW-1 recognized them, and JWW-2 reacted with HPV6, 11, 33 and 23 but JWW-1 did not. Thus

together the two antibodies are complementary with respect to breadth of reactivity and recognize 33/34 clinically relevant HPV types tested (Table 11).

### **JWW-1 and JWW-2 retain neutralizing capacity of their parental antibodies**

We also tested whether the JWW-1 and JWW-2 chimeric antibodies retained neutralizing capacity against HPV16. Additionally, we selected a few HPV genotypes to assess if the antibody's spectrum of strong Western blot reactivity against HPV6, 26, 45 and 58 was consistent with their *in vitro* neutralizing activity. Mouse monoclonal antibody RG-1 was also tested as well as a human IgG control antibody. RG-1 neutralized HPV16 and 26 only, but the EC50 for RG-1 against HPV16 was lower than the more broadly reactive JWW-1 and JWW-2 antibodies. JWW1 neutralized HPV16, 26, 45 and 58 but not HPV6, consistent with prior Western blot data (Table 5) and previous *in vitro* neutralization data for WW1 [13]. JWW-2 was able to neutralize HPV6, 6, 45 and 58, as described for its parental antibody MAb24 [132], but not HPV26, and this spectrum of neutralization was consistent with its strong reactivity by Western blot to L2 of these genotypes (Table 11). Thus JWW-1 and JWW-2 are complementary in terms of their spectrum of neutralizing capabilities (Table 11), and similarly neutralized HPV16 (Figure 20D).

## **DISCUSSION**

L2-based vaccination is strongly protective against experimental viral challenge in numerous pre-clinical models [119, 120, 123, 201, 333, 334]. A limited number of early phase TA-CIN vaccine trials suggest that HPV16 L2 is also immunogenic in patients although the response is much weaker than for L1 VLP [292, 296, 335] suggesting the need for an adjuvant. However, the immune response to L2 elicited by infection with HPV and its associated anogenital neoplasia is poorly understood, and the few existing studies vary widely in their estimates of sero-prevalence. Here, using a two-step screening approach on serum specimens from a total of 1078 patients entered in four prior studies, we find that the prevalence of antibodies toward HPV16 L2 is very



low (<1%); only 7 out of 1078 patients (6 from the CATCH study and 1 from the HPV16+ CIN2/3 group) showed reactivity against HPV16 L2 in both ELISA and Western Blot (Table 8). We also note that once HPV16+ high grade neoplasia has been established, neither the topical treatment of high grade VIN lesions with imiquimod nor lesion ablation with PDT elicited a detectable systemic L2 antibody response (Figure 18C), suggesting that local inflammation was not a sufficient trigger, although this may also reflect low or absence of L2 expression in VIN2/3. In contrast, when these VIN patients were subsequently vaccinated with the TA-CIN, an L2 antibody response was detected in 12/19, even without the use of an adjuvant. This suggests that the failure of most HPV-infected patients to generate an L2-antibody response does not reflect an inability to do so, but rather inadequate presentation of L2 to the immune system.

The use of the Western blot approach was important to verify reactivity as the HPV16 L2 antigen used in the ELISA was generated by recombinant bacterial expression and patients may have pre-existing antibody to bacterial proteins. Indeed, 12/18 CATCH patient sera were positive in the HPV16 L2 ELISA but failed to react with L2 by Western blot analysis, suggesting their reactivity with low level bacterial impurities in the HPV16 L2 preparations. This issue may explain in part the wide variation in the sero-prevalence of L2 antibodies reported in the literature, as many studies did not use a second complementary assay to verify reactivity.

None of the 6 patients from the CATCH study with HPV16 L2-specific antibodies were positive for high risk HPV DNA by Hybrid Capture II in contemporaneously collected cervical cytologic specimens. These subjects had either normal Pap smears or ASCUS (atypical squamous cells of undetermined significance) cytology (Table 7). The single high grade CIN patient with HPV16 L2-reactive serum was diagnosed at study entry with HPV16+ CIN2 that spontaneously regressed within 15 weeks (Table 9). Importantly, 6/7 patient sera were non-reactive to HPV16/18 VLP or the pseudovirion ELISAs, suggesting that these patients lacked L1-specific antibodies to epitopes

on the virion surface and that the patients potentially recognized buried epitopes on the virion. These data also imply that HPV infection generates a predominantly L1-specific antibody response, or rarely an L2-specific antibody response, but typically not both. Indeed, animal studies suggest that L2 is subdominant to L1 in the context of a VLP. It is possible that the L1 and L2-specific responses are mutually exclusive, but we noted a discrepancy in one CATCH study patient who is part of the 7/1078 (patient 9, Table 8) whereby the serum reacted with HPV16 L1 VLP as well as HPV16 L2 by ELISA and HPV16 L2 by Western blot. However the serum failed to neutralize HPV16 pseudovirions. One possible explanation is the serum contains antibody to either insect proteins or denatured L1 present in the HPV16 L1 VLP preparations that are generated in Sf9 insect cells.

Assuming the HPV16 L2 antibody responses were induced by HPV16 infections, they were detectable only in patients who cleared their infections since none of the 6 CATCH study patients with HPV16 L2 antibodies was HC2+, and the HPV16+ CIN2 patient cleared her HPV16 infection. Another possible reason why the six positive patients in the CATCH study were currently HC2- could be that the HPV16 L2 reactivity reflects the induction of a cross-reactive L2 antibody induced by infection by HPV type(s) other than those detected by the HC2 test.

Although these 7 patient sera were reactive towards HPV16 full length L2 ELISA and Western blot, none were detectably neutralizing for HPV16 ( $EC_{50} < 1:50$ ) using the standard *in vitro* neutralization assay (L1-PBNA). One explanation for this discrepancy is that ELISA using bacterially expressed L2 and Western blots primarily present linear epitopes, while L2 neutralizing responses require a specific conformation. However, the neutralizing epitopes of L2 are typically linear, suggesting this explanation is unlikely. Further, these sera also failed to react detectably with HPV16 L2 residues 11-88 (in the L2 $\alpha$ 11-88x5 antigen) or HPV16 pseudovirions (which contain full length L2) by ELISA (Table 8). Taken together, these observations suggest

that the L2 antibodies in these serum samples are directed towards non-neutralizing epitopes at the C-terminus of HPV16 L2 protein, a region mostly buried below the virion surface [30]. This suggestion is also based on prior serologic studies mapping epitopes of HPV L2 and/or L2 antibody prevalence have reported immune reactivity towards L2 aa 110-210 and aa 391-402 [319-322]. In some studies, L2 antibodies were weakly associated with HPV infection, CIN3 or cervical cancer whilst others have reported an L2 antibody seroprevalence of 20-30% in genital wart and cervical dysplasia patients [323-325]. Here we observed a much lower HPV16 L2 antibody sero-prevalence (<1%). However, we note that in the above-mentioned studies either Western blots or ELISA with bacterially-expressed L2 were solely utilized for screening in several previous studies. In contrast, here, while a two-step validation approach was used to minimize potential false positives due to bacterial protein-specific serum antibody, it may compromise assay sensitivity.

The current WHO guidelines for HPV VLP vaccines requires the comparison of neutralizing antibodies induced from HPV vaccines to responses from infection for specific HPV types [336]. Although there are WHO international standards for HPV16 and HPV18 antibodies based upon pooled sera from infected patients, they are unsuitable for validation of L2-specific antibody responses as they failed to react to HPV16 L2 by Western blot or ELISA (data not shown). As these WHO standards contain only type-specific anti-L1 antibodies, an alternate serological standard for human serum antibody responses to L2 is still needed.

Despite reactivity by both HPV16 L2 ELISA and Western blot, not one of the seven sera from infected patients were detectably neutralizing. While the sera of patients vaccinated with TA-CIN could potentially be pooled and utilized as a standard, the limited amount and value of the remaining sera renders this approach impractical. An alternate strategy to derive a sustainable and generalizable L2-specific neutralizing antibody standard is to synthesize chimeric human L2-

specific monoclonal antibodies based upon well-defined neutralizing rodent monoclonal antibodies. To this end, we created two chimeric human monoclonal antibodies, JWW-1 and JWW-2, which retained respectively the L2 a.a. 18-32 and 58-64 epitope specificity and neutralizing activity of their parental rodent monoclonal antibodies. Both JWW-1 and JWW-2 could be detected using anti-human IgG secondary antibody, demonstrating its potential utility as a standard reagent for immunoassay validation when testing human sera by ELISA. JWW-1 was reactive to many clinically relevant mucosal and cutaneous HPV types (29/34), but, like WW1, it does not bind to L2 of two common low risk types, HPV6 and HPV11, or to high risk HPV33 (Table 10). In contrast, JWW-2 is able to bind to these three important types but not certain hrHPV types such as HPV26 and 51 that are recognized by JWW-1 (Table 10). However, the avidity of each antibody to L2 of different HPV types varies significantly, as can be seen from the *in vitro* neutralization data for example (Table 11). Taken together, the ability of these antibodies to recognize different epitopes on L2 and complement in terms of their spectrum of reactivity suggests the utility of both antibodies as potential reference standards. Importantly, both monoclonal antibodies individually were neutralizing to a variety of clinically relevant HPV types (Table 11) thereby showing their utility for neutralization studies and their functional relevance. The complementary neutralization of diverse HPV types by JWW-1 and JWW-2 suggest the utility of incorporating both of their epitopes in next generation L2-based vaccines.

In conclusion, patients rarely have L2-specific serum antibody even in populations at high risk for HPV infection in which candidate L2-based preventive HPV vaccines would be tested. Secondly, because spontaneous responses are rare and may contain neutralizing L1-specific antibody, the creation of an international standard for L2-specific neutralizing antibody based upon finite pools of sera from infected patients would be challenging. Further, the natural L2-specific sera identified in our study were not detectably neutralizing. By contrast, human chimeric monoclonal antibodies JWW-1 and JWW-2 are broadly reactive, neutralizing and readily replenished, and

thus represent a promising alternative as antibody standards to validate in future L2-specific immunologic assays, including *in vitro* neutralization, for sero-epidemiologic or L2 vaccine studies.

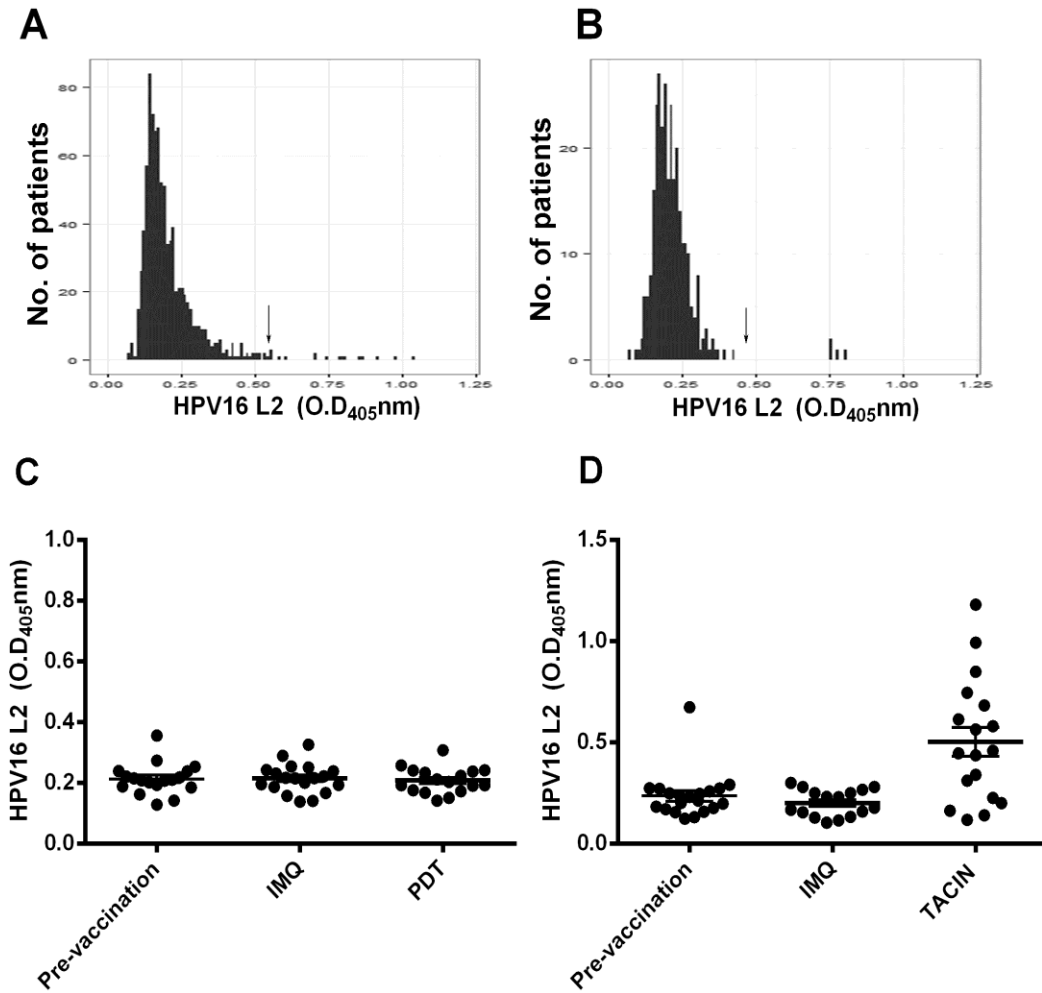


Figure 18. **HPV16 L2-specific serum antibodies in VIN patients**

Optical density values plotted for HPV16 L2 ELISA measurements of the serological responses of: (A) previously unscreened *CATCH* study patients (n=880) of which 10.3% were positive for high risk HPV, (B) high grade CIN patients (n=160) with 1-4 visits (n=298), (C) high grade HPV+ VIN patients (n=19) that underwent 10 weeks of topical imiquimod therapy, which activates TLR7-dependent innate responses (IMQ) before being treated with photo-dynamic therapy (PDT) in a phase II study [329], and (D) high grade HPV+ VIN patients (n=19) that underwent 10 weeks of topical imiquimod therapy (INN) followed by three vaccinations with 125 µg TACIN at monthly intervals in a prior phase II study prior to serum collection [296]. The absorbance values to the right of the arrow in A and B (mean + 3 s.d. cut-off) were considered

putative positive responses and re-screened in a Western blot assay. For figure 18A, 18 data points corresponding to 18 different patients were above the cut off (arrow). For Figure 18B, the 4 data points larger than the cut-off (arrow) corresponds to four serum samples collected for each visit of a single HPV16+ CIN2 patient and each was also reactive for HPV16 L2 by Western blot.

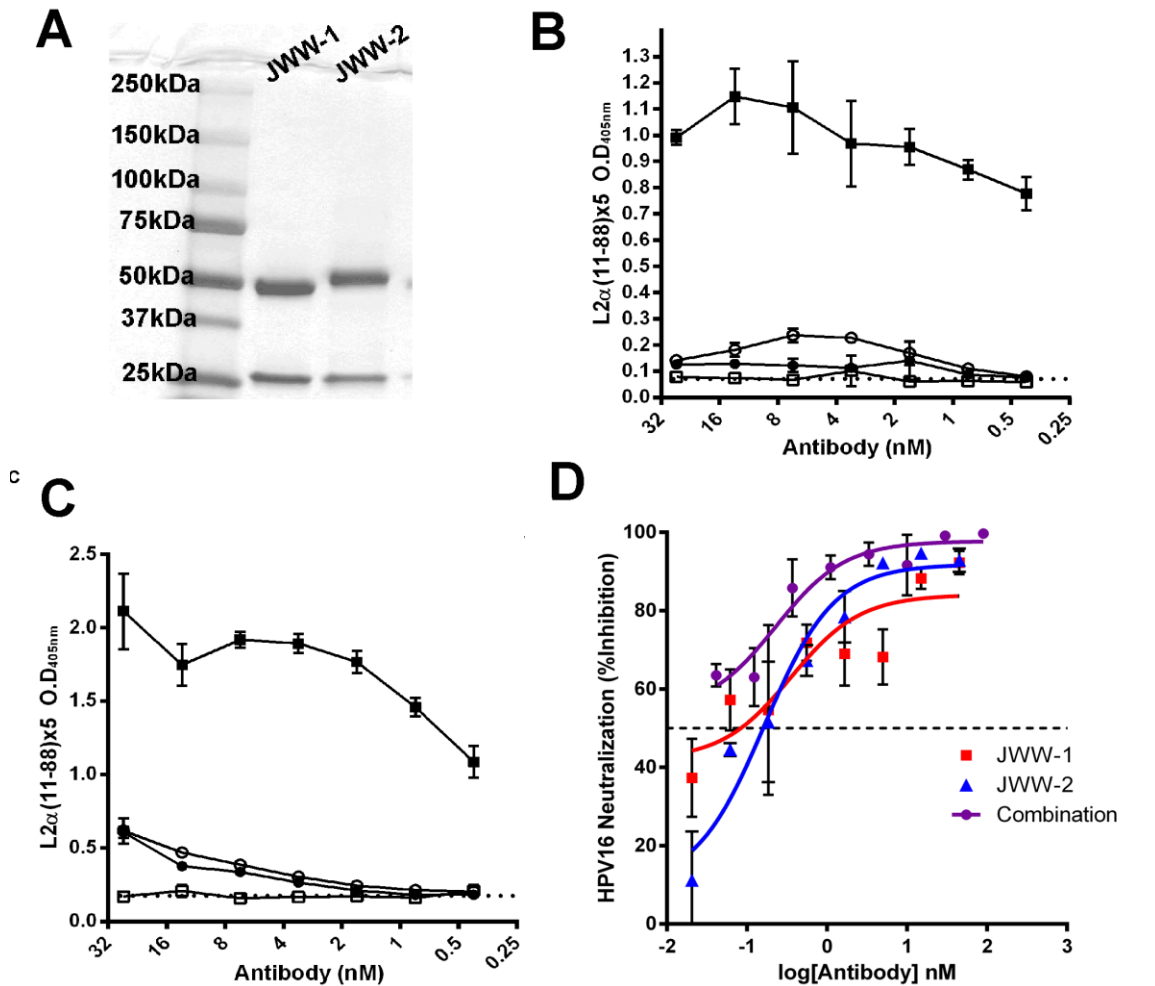


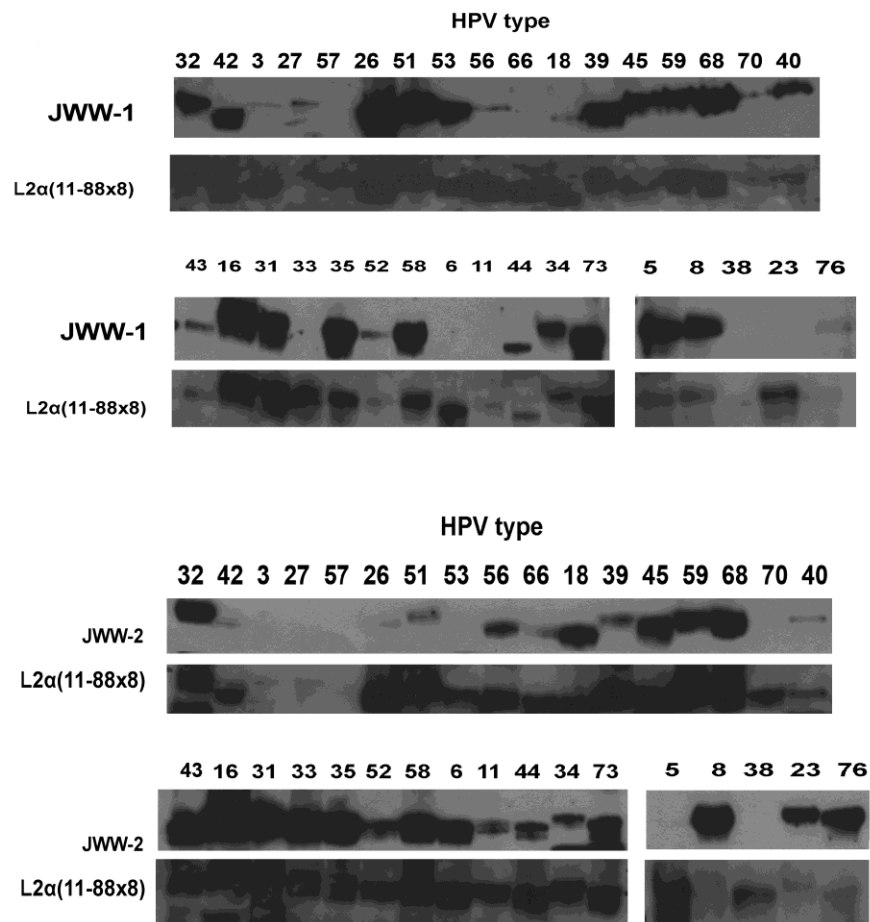
Figure 19. **JWW-1 and JWW-2 are human chimeric, L2-specific neutralizing monoclonal antibodies**

Coomassie blue-stained SDS-PAGE gel analysis of JWW-1 and JWW-2 monoclonal antibodies.

(B) L2 $\alpha$  (11-88)x5 multimer ELISA using purified JWW-1 (closed squares), rat monoclonal WW-1 (open squares), WHO standard HPV16 serum 05/134 (closed circles), and serum from an HPV16+ CIN2 patient (visit 1, open circles) containing HPV16 L2-reactive antibodies. (C) L2 $\alpha$  (11-88)x5 multimer ELISA using purified JWW-2 (closed squares), mouse monoclonal MAb24b (open squares), WHO standard HPV16 serum 05/134 (closed circles), and serum from an HPV16+ CIN2 patient (visit 1, open circles) containing HPV16 L2-reactive antibodies. (D)



Neutralization assay comparing JWW-1 and JWW-2 and a 1:1 combination of each with starting molarity of 45nM. Neutralization is compared to the total antibody concentration.



**Figure 20.** Western blot reactivity of JWW-1, JWW-2 and rabbit antiserum to L2α(11-88x8) against L2 in pseudovirion preparations of 34 clinically-relevant alpha and beta HPV types.

**Table 7.** Characteristics of 880 CATCH study patients who provided sample for the serology study. \* This 38% sample was younger than the total enrolled population ( $p < 0.01$ ) but did not differ by enrollment cytology result ( $p = 0.18$ ) or HR-HPV result ( $p = 0.56$ ).

<b>Characteristic</b>	<b>N (%)*</b>
<b>Age</b>	
30-34	174 (19.6)
35-39	121 (13.6)
40-44	95 (37.1)
45-49	54 (6.1)
50-54	50 (5.6)
55-59	29 (3.3)
60+	36 (4.0)
Not reported	34 (3.8)
<b>Cytology</b>	
Normal	763 (86.5)
ASCUS or more severe	119 (13.5)
<b>HPV</b>	
Negative	787 (89.2)
Positive	95 (10.8)
<b>Histology</b>	
Normal	222 (24.9)
CIN1	2 (0.22)
CIN2	5 (0.56)
CIN3	3 (0.34)
Cancer	4 (0.45)
Other	6 (0.67)
No biopsy performed	648 (72.8)

**Table 8.** Summary of L2 ELISA screened patient sera. Shaded rows show the patients (n=7/19) whose sera displayed reactivity for full length HPV16 L2 by both ELISA and Western blot. N\* indicates sera was limiting and the Western blot could only be done at a dilution of 1:5,000. Sera numbers 1-18 were from the CATCH study near Hyderabad, AP, India, and serum 19 was from the CIN cohort study at Johns Hopkins University, Baltimore, MD USA. N.D indicates not done.

PATIENT		ELISA Antigen									
CATCH STUDY PATIENTS	No.	Visit	HPV 16 L2	HPV16 VLP	HPV18 VLP	HPV31 PsV	HPV58 PsV	HPV45 PsV	L2α(11-88)×5	Western Blot	
	1	1	1.039	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Y
	2	1	0.979	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Y
	3	1	0.910	Neg	Neg	Neg	Neg	Neg	Neg	N.D	N
	4	1	0.869	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Y
	5	1	0.850	Neg	Neg	Neg	Neg	Neg	Neg	N.D	N
	6	1	0.804	1.087	Neg	Neg	Neg	Neg	Neg	N.D	N
	7	1	0.797	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Y
	8	1	0.783	Neg	Neg	Neg	Neg	Neg	Neg	N.D	N
	9	1	0.747	1.170	Neg	Neg	Neg	Neg	Neg	Neg	Y
	10	1	0.701	Neg	Neg	Neg	Neg	Neg	0.728	N.D	N
	11	1	0.700	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Y

	12	1	0.605	0.796	Neg	Neg	Neg	Neg	N.D	N
	13	1	0.587	Neg	Neg	0.821	0.413	1.095	N.D	N*
	14	1	0.558	Neg	Neg	Neg	Neg	Neg	N.D	N
	15	1	0.553	Neg	Neg	Neg	Neg	Neg	N.D	N
	16	1	0.550	0.432	Neg	Neg	Neg	Neg	N.D	N*
	17	1	0.548	Neg	Neg	0.606	Neg	0.966	N.D	N
	18	1	0.534	0.703	0.707	Neg	Neg	Neg	N.D	N
<b>CIN PATIENTS</b>	19A	1	0.752	N.D	N.D	N.D	N.D	N.D	Neg	Y
	19B	2	0.803	N.D	N.D	N.D	N.D	N.D	Neg	Y
	19C	3	0.776	N.D	N.D	N.D	N.D	N.D	Neg	Y
	19D	4	0.755	N.D	N.D	N.D	N.D	N.D	Neg	Y

**Table 9.** Characteristics of the cohort of 160 women with high grade CIN enrolled in a natural history study [205] and treated at Johns Hopkins University, Baltimore, MD USA. Serum samples were collected at the screening visit as well as subsequent treatment and follow up visits when available.

<b>Diagnosis</b>	<b>Number (%) of patients</b>
HGSIL	132 (82.5)
Benign/CCSM	11 (6.9)
ASCUS	2 (1.3)
ASC-H	2 (1.3)
AIM	1 (0.6)
LSIL-H	1 (0.6)
LSIL	11 (6.9)
<b>HPV16 status (HGSILs only)</b>	<b>Number of patients (% total)</b>
HPV16+	87 (54.4)
HPV16-neg	42 (26.3)
Not done	3 (1.9)
<b>HPV16 status (all patients)</b>	<b>Number of patients</b>
HPV16+	99 (61.9)
HPV16-neg	53 (33.1)
Not done	7 (4.4)
Indeterminate	1 (0.6)
<b>Race/ethnicity</b>	<b>Number of patients</b>
White	109 (68.1)
Black	37 (23.1)
Asian	5 (3.1)
Hispanic	9 (5.6)
<b>Age groups</b>	<b>Number of patients</b>
18-20	16 (10.0)
21-30	86 (53.8)
31-40	39 (24.4)
41-50	13 (8.1)
50 and older	6 (3.8)

**Table 10.** Summary of JWW-1 and JWW-2 Western blot reactivity against L2 of 34 clinically-relevant alpha and beta HPV types. Green=band, Red=No band detected by Western blot of HPV pseudovirion preparations. Western blot data provided in Figure 20.

Sub-family	HPV type	Mucosal/ Cutaneous	Antibody Reactivity		
			JWW1 L2 <sub>18-32</sub>	JWW-2 L2 <sub>58-62</sub>	L2 $\alpha$ (11-88)X8 Rabbit Sera
$\alpha$ 1	32	M	Green	Green	Green
	42	M	Green	Red	Green
$\alpha$ 2	3	C/M	Green	Red	Green
$\alpha$ 5	26	M	Green	Red	Green
	51	M	Green	Red	Green
$\alpha$ 6	53	M	Green	Red	Green
	56	M	Green	Green	Green
	66	M	Green	Red	Green
$\alpha$ 7	18	M	Green	Green	Green
	39	M	Green	Green	Green
	45	M	Green	Green	Green
	59	M	Green	Green	Green
	68	M	Green	Green	Green
	70	M	Green	Red	Green
$\alpha$ 8	40	M/C	Green	Green	Green
	43	M/C	Green	Green	Green
$\alpha$ 9	16	M	Green	Green	Green
	31	M	Green	Green	Green
	33	M	Red	Green	Green
	35	M	Green	Green	Green
	52	M	Green	Green	Green
	58	M	Green	Green	Green
$\alpha$ 10	6	M	Red	Green	Green
	11	M	Red	Green	Green
	44	M	Green	Green	Green
$\alpha$ 11	34	M	Green	Green	Green
	73	M	Green	Green	Green
$\beta$ 1	5	C	Green	Red	Green
	8	C	Green	Green	Green
$\beta$ 2	38	C	Red	Red	Green
	23	C	Red	Green	Green
$\beta$ 3	76	C	Green	Green	Green

**Table 11. In vitro neutralization capacity of purified L2 monoclonal antibodies.** Shown is a summary of IC50 [95% Confidence Interval] nM of purified monoclonal antibodies RG1, JWW1, JWW-2 and a negative control human IgG determined using the FC-PBNA against HPV 6/16/26/45 and 58 pseudovirions using a starting antibody concentration of 200nM (30µg/mL).

<b>HPV</b>	<b>Monoclonal Antibody</b>	<b>IC50 [95% C.I.] nM</b>
HPV 6 (Luciferase Reporter)	RG-1	>200 [N/A]
	JWW-1	>200 [N/A]
	JWW-2	160.3 [21.55-1191.9]
	human IgG control	>200 [N/A]
HPV 16 (Luciferase Reporter)	RG-1	<0.27 [N/A]
	JWW-1	<0.27 [N/A]
	JWW-2	4.75 [3.27-6.88]
	human IgG control	>200 [N/A]
HPV 26 (Luciferase Reporter)	RG-1	3.24 [2.17-4.86]
	JWW-1	<0.27 [N/A]
	JWW-2	>200 [N/A]
	human IgG control	>200 [N/A]
HPV 45 (Luciferase Reporter)	RG-1	>200 [N/A]
	JWW-1	6.57 [2.28-18.6]
	JWW-2	71.45 [5.22-978]
	human IgG control	>200 [N/A]
HPV 58 (Luciferase Reporter)	RG-1	>200 [N/A]
	JWW-1	67.29 [10.1-506.2]
	JWW-2	75.47 [11.75-484.8]
	Rat IgG control	>200 [N/A]



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#### IV. Roles of Fc domain and exudation in L2 antibody-mediated protection against Human Papillomavirus

##### ABSTRACT

To examine how L2-specific neutralizing IgG protects broadly against genital human papillomavirus (HPV), rats were immunized with L2 multimer and two cross-neutralizing monoclonal antibodies (MAbs), WW1 and S10, were generated. The linear epitope of WW1 resides within L2 residues 17-36, like the mouse MAb RG1. However WW1 bound L2 of 29/34 HPV genotypes, whereas RG1 reacted with only 13/34. S10 recognized a conformational epitope within residues 68-83 of HPV6, 11, 44 and 58. Both WW1 IgG and (Fab')<sub>2</sub> fragments bound to HPV16 pseudovirions similarly, but the IgG better protected mice against vaginal HPV16 challenge (94.8% at 25nM) than (Fab')<sub>2</sub> (only 78.8% at 75nM). Passive transfer of WW1 IgG was similarly protective in wild type and FcRn-deficient mice, suggesting the weaker protection by WW1 (Fab')<sub>2</sub> is not due to impaired Fc-mediated transcytosis. Furthermore, local microtrauma or administration of nonoxynol-9, which facilitates HPV infection, releases serum IgG to the genital tract, suggesting Fc-independent exudation of serum IgG occurs locally during viral challenge. Depletion of neutrophils and macrophages reduced protection of mice via passive transfer of WW1 IgG (83nM) from 93% to 82% suggesting Fc facilitates phagocytosis. However, *in vitro* neutralization by WW1 IgG was ~10-fold more potent than for (Fab')<sub>2</sub>, implying an additional role for Fc, but this was not related to the TRIM21-Antibody-Dependent Intracellular Neutralization (ADIN) pathway. In conclusion, the Fc of WW1 both opsonizes extracellular virions and enhances neutralization in the absence of phagocytes, but neither FcRn-mediated transport nor TRIM21-dependent intracellular neutralization mechanisms contribute to protection from genital challenge.

## INTRODUCTION

Over 200 types of HPV have been described, and these small DNA tumor viruses are generally classified into either cutaneous or mucosal types based on their specific tissue tropism [337]. The mucosal types are then further sub-categorized into the ‘Low Risk’ types associated with genital warts (e.g HPV6 and HPV11) and fifteen ‘High Risk’ types which have oncogenic potential which of particular interest is HPV16, which accounts for 50% of all cervical cancer cases and approximately 90% of all HPV-associated cancers at other anatomical sites [338, 339].

There are currently two commercial HPV preventive vaccines, Gardasil® and Cervarix®, which safely confer durable and robust protection against their targeted HPV types [284]. These vaccines are based upon highly immunogenic virus-like particles (VLP) assembled from the major capsid protein L1 [24]. Both Gardasil® and Cervarix® contain L1 VLPs of HPV16 and HPV18, genotypes that account for approximately 50 and 20% of all cervical cancer cases respectively. Gardasil® also contains HPV6 and HPV11 VLPs as these two genotypes cause ~90% of genital warts. Both vaccines can elicit a strong type-restricted neutralizing response against the HPV type from which the L1 was derived [283, 340-343]. Although some cross-neutralization and protection against closely related types (e.g HPV31 and HPV45) occurs [283], their limited breadth of protection has spurred efforts to produce new HPV vaccines also effective against the other dozen oncogenic HPV types.

The remaining component of the viral coat is the minor capsid protein L2 which mediates endosome escape and, in the absence of L1, trafficks to the nucleus with the viral genome (reviewed in [6]). The amino terminal sequence of L2 possesses several motifs that are both functional in viral infection and conserved, broadly neutralizing epitopes [120, 287, 333], including residues 17-36 recognized by MAbs RG1 [68], and residues 58-81 and 108-120 mapped with antisera and MAbs [41, 132, 287, 333, 335]. Passive transfer studies suggest that

neutralizing antibodies specific for either L1 VLPs or L2 are sufficient to mediate protection in animal challenge models. Vaccination with L2 generates lower titer but more broadly neutralizing antibodies than L1 VLPs [126], raising questions about the durability of L2-specific immunity [41, 344]. To enhance the titer of the neutralizing serum antibody response, several approaches have been tested, including use of strong adjuvants and concatenation of L2 epitopes and/or repetitive display on macromolecular scaffolds such as viral VLP platforms to increase epitope density [6, 7, 41, 318, 345]. While all these methods have succeeded to an extent, the L2-specific titers are still inferior to L1 VLP vaccines. Nevertheless, remarkably low titers are sufficient via passive transfer to mediate full protection against viral challenge in the murine genital tract [18, 294, 331].

The mechanism of antibody-mediated protection is incompletely understood, including how neutralizing serum IgG in the systemic circulation might reach the viral inoculum in the reproductive tract. This may occur via active transcytosis into the genital tract mediated by the neonatal Fc receptor (FcRn), and/or possibly exudation associated with microtrauma at the site of infection. Indeed, experimental infection of the murine genital tract requires epithelial trauma (or disruption by pre-treatment with nonoxynol-9) such that virions can bind to heparin-sulfate proteoglycans on the basement membrane (BM). Association with the BM induces conformational changes in the capsid which permit association of virions with the basal keratinocyte and infection. Importantly, this conformation change renders certain L2 epitopes, including residues 17-36 recognized by RG1, accessible on the capsid surface to permit neutralization [44]. High concentrations of L1 VLP-specific antibodies block the initial association to the BM, whereas lower levels permit BM association but block the binding of virions to basal keratinocytes [294], as is also seen for neutralization by L2-specific antibody (for review see [346]). Antibody-bound virions on the BM are cleared over 18h, and this was associated with neutrophil infiltrates, suggestive of Fc-mediated opsonisation and phagocytosis.

However, the contribution of neutrophils to protection was not directly tested and this cannot be the sole mechanism of protection since L2 antibodies are potently neutralizing *in vitro* in the absence of neutrophils.

L2-specific neutralizing antibodies do not prevent virion binding to cultured cell monolayers [44, 84]. Day *et al* found that following binding, the L2 antibody-virion complex slowly accumulated on the ECM, but did not enter cells. However in another study, uptake was only partially inhibited. Rather, the bulk of L2 antibody-bound virions accumulated in the perinuclear region in large lamellar bodies and lysosome-like multi-vesicular bodies suggesting that neutralization may occur by preventing L2:viral DNA egress from the vesicular compartment [287]. Alternatively, L2 antibody-dependent neutralization may occur in the cytoplasm by a mechanism recently described for several viruses as a final defense to counter the ‘persistent fraction’ of virions bound by otherwise non-neutralizing antibodies that escape into the cytoplasm [347, 348]. In this mechanism, the Fc of the antibody-virus complex is bound with high affinity by the cytosolic Fc receptor TRIM21. TRIM21 then catalyzes the attachment of K63-linked polyubiquitinated chains which both commits the antibody-virion complex for proteasomal degradation and activates an innate response presumably to combat early viral replication [347-350]. Since L2 traffics to the nucleus during infection, this TRIM21 Fc receptor-dependent mechanism might contribute to sterilizing immunity provided by L2-specific antibody. Here, we generated new L2-specific, broadly neutralizing monoclonal antibodies, and re-examined the mechanisms of L2 antibody-mediated protection.

## **MATERIALS AND METHODS**

### **Ethics Statement**

Animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and with the prior approval of the Animal Care and Use Committee of Johns Hopkins University.

### **Cell Cultures.**

293TT, 293TTF and LoVoT cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum, 1X penicillin, and streptomycin, 1X Non-essential amino acids, 1X Sodium Pyruvate (Gibco, Life Technologies, Grand Island NY). 2 µg/ml and 200 µg/ml of puromycin and hygromycin were added to 293TTF and LoVoT respectively to maintain expression of furin/T-antigen expression.

### **Generation of HPV pseudoviruses (PsV)**

Pseudoviruses (PsV) and furin-cleaved Pseudoviruses (fcPsV) were generated as previously described in (<http://home.ccr.cancer.gov/Lco/pseudovirusproduction.htm>) and [13] respectively. The 34 medically relevant HPV plasmids utilized in this study are described here ([351]) and can be obtained either from <http://home.ccr.cancer.gov/LCO/> or Addgene ([http://www.addgene.org/Richard\\_Roden/](http://www.addgene.org/Richard_Roden/))

### **Recombinant Proteins, Peptides, Monoclonal Antibodies and Serum Samples.**

The anti-L2 mouse MAb RG1 was previously generated as described [68]. Full length HPV L2 from HPV6/11/16/18/31 and L2 $\alpha$ (11-88 $\times$ 8) antigen were cloned in pProEx and pET28 plasmid respectively and purified as previously described [344]. HPV anti-L1 VLP or anti-L2 rabbit sera that used in this study were previously generated from experimentally immunized rabbits with different type of L1 virus-like particles, PsV or  $\alpha$ 11-88 $\times$ 8 L2 antigens respectively. These sera

have been previously tested for the presence of antibodies to HPV proteins by HPV *in vitro* neutralization assay or by Western blot [344].

### **Development of Rat Monoclonal Antibodies to L2 (WW1 and S10), and generation of (Fab')<sub>2</sub> fragments**

Two Lewis rats were immunized five times with 50 µg of L2α11-88x8 formulated with TiterMax® (TiterMax USA, Inc.) adjuvant. One week following the final immunization, splenocytes were harvested and fused with SP2/0 myeloma cells using polyethylene glycol (PEG). Supernatants from wells containing viable hybridomas were screened at 10 to 14 days after fusion by ELISA using bacterially expressed 6His-tagged L2, L2α(11-88x8) or full length L2 peptide derived from HPV type 11, 16, 18, or 31, as a coating antigen. Selected positive clones were re-cloned three times by limiting dilution and antibodies secreted were purified through protein G sepharose (GE healthcare). Following purification, purified WW1 and S10 rat MAbs were either cleaved with pepsin-Sepharose to generate (Fab')<sub>2</sub> fragments by using kits according to product instructions (Pierce, ThermoScientific). Commassie blue staining and ELISA was performed to assess purity of antibody fragments and to estimate the amount of whole antibodies and their binding properties. The rat anti-CD4 antibody (clone GK1.5) was utilized as the non-specific rat IgG control antibody (BioXCell, West Lebanon, NH).

### **Characterization of rat MAbs**

Determination of the Ig subclass of the rat MAbs was performed using a commercial isotyping kits (BD biosciences, CA). Commercial overlapping 15mer peptide arrays (Mimotope; Minneapolis, MN) comprising the peptides within 11-88 regions of 15 different HPV types were coated onto ELISA plates for the binding assay. Hybridoma supernatants (1:200) or purified antibodies (1:5000; from stock with concentration between 1 to 2 mg/ml) were added to the blocked peptide array plates and incubated for 1 h at 37°C. Plates were washed three times with

PBS containing Tween-20 again and then incubated with HRP conjugated goat anti-rat IgG for 60 min at 37°C. Unbound MAb was then washed away with 0.01% Tween 20 in PBS and signal detected with ABTS (Roche, Basel Switzerland). To determine a relationship between HPV L2 cysteine residues and monoclonal antibodies binding, blocked plates were coated with HPV virions that had either wild type L2 or mutant HPV16 L2 [352] and the processed the same as the above peptide array mapping procedure.

### **Western Blot Analysis**

For the detection of the L2 in pseudoviruses, samples normalized to L1 protein (500 ng) were boiled for 5 min in reducing gel sample buffer and analyzed by SDS-PAGE analysis using 4-20% pre-casted Tris-HCL gels (Bio-rad, CA). Proteins were electro-transferred to PVDF membranes (Bio-Rad, CA) and the PVDF membranes were blocked with 5% skim milk in PBST (PBS containing 0.1% Tween 20) for 1-2 hours before being incubated with the appropriate MAbs or serum samples at a 1:5000 dilution. After primary incubation, blots were washed with PBST three times for ten minutes each before addition of either HRP-conjugated goat anti-rat, sheep anti-mouse IgG (GE health) or sheep anti-rabbit IgG (GE health) secondary antibodies (1:10,000) respectively. Secondary antibody incubation was done for 1-2 hours at room temperature. Membranes were then washed three times with PBST three times 10 mins each time. Chemiluminescence substrate was added to develop the membranes.

### **ELISA**

HPV PsV antigens were normalized to 500 ng/well based on L1 amount using both commassie blue staining and BCA assay (Thermo Scientific). L2 antigens, amino acids 17-38 derived from HPV type 6, 16, 31, 35, 45 and 58 were chemically synthesized as a peptide were coated onto an ELISA plate (MaxiSorp; Thermo Scientific) at an amount of 100 ng in 50 µl per well by incubating at 4°C overnight. For comparing the binding capacity of whole IgG, Fab and F(ab')<sub>2</sub>,



HPV16 L2 peptide 18-37 (500 ng/well) or PsV were used as coating antigens and coated at 37°C for 2 h. Coated plates were blocked with PBS-BSA (1x PBS supplemented with 1% BSA) at 37°C for 1 to 2 h and then incubated with 50 µl of the diluted monoclonal antibodies or serum at ambient temperature for 1 h. Plates were then washed three times with PBST (PBS with 0.01% Tween 20), and bound antibodies were detected with horseradish peroxidase-conjugated protein G (HRP protein G) (Pierce, Rockford IL), biotin mouse anti-rat IgG2a or Ig kappa light chain monoclonal antibodies followed by staining with 2,2'-azinobis[3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS) substrate (Roche) or peroxidase labeled streptavidin at 1:1250 (0.1 mg/ml; KPL, Gaithersburg MD) before adding of substrate. The cutoff OD value ( $OD_{\text{cutoff}}$ ) for the ELISA assay was set at three times the average absorbance detected from no primary antibody control wells. The highest dilution of serum sample that had an OD value equal to or above the  $OD_{\text{cutoff}}$  was defined as the endpoint ELISA titer and expressed as its reciprocal value or antibody concentration.

### **HPV *in vitro* neutralization**

15,000 293TT or LoVoT cells/well were pre-plated in a 96 well plate. 24 hours later, on a separate plate, monoclonal antibodies WW1, RG1, S10, rat control IgG were serially diluted two-fold in culture medium across the plate. Subsequently, an equal volume of HPV pseudovirions containing luciferase reporter genes was added to the plate and this mixture was incubated at 37°C for 2 hr and added to either the pre-plated 293TT or LoVoT cells. The cells were then incubated for 72 h. To measure neutralization, culture media were removed from each well and 1X Cell Culture Lysis Reagent (Promega, Madison WI) was added to lyse the cell monolayer for 15 min at room temperature on a rocking platform. The entire lysate from each well was transferred to a black 96-well plate followed by the addition of 50 µl/well of 1x luciferase substrate (GloMax<sup>®</sup>-Multi Detection System, Promega, Madison WI). Neutralization titer is determined as the reciprocal of the dilution that causes 50% reduction in luciferase activity. The

titer molar concentration was then subsequently back-calculated into nM as all mAbs were used were at a starting concentration of 1666 nM.

**Passive transfer of antibodies and *in vivo* PsV vaginal challenge using cytobrush or N9 method**

4-6 weeks old female Balb/c mice purchased from NCI (Frederick) were injected subcutaneously with 3 mg of medroxyprogesterone (Depo-Provera; Pfizer) to synchronize their estrus cycles. Three days later, the mice in groups of five or ten were passive immunized intraperitoneally with either purified mAbs, control IgGs or HPV L1 VLP or L2-specific rabbit antisera as a positive control. 24 hours after passive transfer, each mouse was given a HPV PsV challenge dose of 40 $\mu$ L comprised of 20 $\mu$ L PsV mixed with 20 $\mu$ L of 3% carboxymethyl cellulose (CMC). Half of the challenge dose (20 $\mu$ L) was injected into the mouse vaginal vault, followed by insertion of a cytobrush cell collector that was turned both clockwise and counter-clockwise 15 times to induce trauma. After removal of the cytobrush, the remaining half of the inoculum was deposited in the vagina while the mice were anesthetized. For experiments using the N9 method, 24 hours following passive immunization, mice were pre-treated with 4% N9 while being anesthetized and the entire virus inoculum dosage (40  $\mu$ L) was delivered into the vaginal vault. 72 hours after HPV PsV challenge, the mice were again anesthetized and 20  $\mu$ L of luciferin (7.8 mg/mL in water) was deposited in the vaginal vault. Luciferase signals were acquired for 10 min with a Xenogen IVIS 100 imager, and analysis was performed with Living Image 2.0 software. For wound neutrophil and macrophage depletion, mice were treated with 100 $\mu$ g/mouse of the rat monoclonal RB6-8C5 (BioXCell, West Lebanon, NH) for 3 consecutive days followed by administration of this antibody once a week. Depletion was ensured via flow cytometry prior to passive transfer and HPV16 PsV challenge studies.

### **Vaginal lavage and ex-vivo detection of L2-specific MAb**

Rat L2-specific MAb was passively transferred into Balb/c, C57BL/6 or FcRn knockout mice and the amount presence in the vaginal tract were determined with L2-specific ELISA. Mice were synchronized with 3 mg of medroxyprogesterone (Depo-Provera; Pfizer) at 4 days before passive transfer with 100 µg of rat MAbs. Mice were anaesthetized, then cytobrush or N-9 treatment were performed simultaneously along with antibody transfer or done at 6 h earlier. Lavages were collected at 6 or 12 h following passive transferring of MAb. At the indicated time, mouse was anaesthetized, deposited twice with 500 µL of 1x PBS, then lavage fluids were collected and keep at -20°C for analysis with L2-specific ELISA.

### **Bio-informatical and statistical analysis**

Data were analyzed with either Mann-Whitney U-test or the Kruskal-Wallis test using GraphPad Prism 6.0. Sequences of HPV L2 types were obtained from PaVE, PapillomaVirus Episteme (<http://pave.niaid.nih.gov/#home>) and analyzed using the UCSF chimera package (<http://www.cgl.ucsf.edu/chimera>). Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS P41-GM103311).

## **RESULTS**

### **Generation of L2-specific cross-neutralizing MAbs, WW1 and S10**

Previous studies showed vaccination of mice with a L2  $\alpha$ 11-88x8 fusion protein (comprising residues 11-88 of HPV types 6, 16, 18, 31, 39, 51, 56, 73) provided robust protection against vaginal challenge with 11 clinically relevant genital HPV genotypes [8]. Rabbit antisera to L2 $\alpha$ 11-88x8 also neutralized 34 clinically relevant HPV types from the alpha and beta families *in vitro* [351] and was protective via passive transfer [8]. Therefore, to generate new more broadly neutralizing L2-specific MAbs, we vaccinated Lewis rats with the same L2  $\alpha$ 11-88x8 antigen.

ELISA screening of hybridoma supernatants identified two rat IgG2a MAbs, named WW1 and S10. A second round of ELISA screening using full length L2 from HPV6, HPV11, HPV16, HPV18, or HPV31 as coating antigen showed that WW1 reacted with HPV16/18/31 L2 but not HPV6 or HPV11 L2, whereas S10 reacted with HPV6/11 L2 protein but not with HPV16, 18 or 31 L2 (data not shown). These specificities were further confirmed by Western blot analysis using the same L2 proteins (data not shown). To further test the breadth of WW1 and S10 reactivity, pseudovirion preparations of 34 clinically relevant HPV genotypes from the  $\alpha$  and  $\beta$  families were probed by Western blot, first with MAbs WW1, S10, or RG1, and then, after stripping, with rabbit antiserum to L2  $\alpha$ 11-88x8 as a positive control to confirm the presence of L2 in all of the pseudovirion preparations (Figure 27). S10 only bound to L2 of HPV6, 11, and 44, members of the  $\alpha$ 10 sub-family, as well as HPV58 L2 from  $\alpha$ 9 sub-family (Figure 23A). In contrast, WW1 reacted with L2 of 29 out of the 34 HPV genotypes tested, whereas RG1 recognized L2 in 13 of these 34 pseudovirion preparations (summarized in Figure 21).

### **WW1 recognizes a linear neutralizing epitope within HPV16 L2 17-36**

A 15mer peptide array of L2 peptides within the 11-88 regions of 15 different HPV types each overlapping by 5 residues was utilized to map the MAb epitopes. WW1 recognized only two overlapping L2 peptides comprising residues 13-32 and 18-37 derived from HPV16 L2 as well as the equivalent L2 peptides from HPV types 18, 31, 35, 39, 45, 51, 52, 58, 59 and 73. Since WW1 did not bind to HPV16 L2 peptide aa 23-42, its epitope likely resides within residues 18-32 of HPV16 L2 (LYKTCKQAGTCPPDI). To further examine the role of conserved residues in this 18-32 residue region, we analyzed WW1 via ELISA using HPV16 PsV containing either wild type L2 or several point mutants within this region (Figure 22). Mutation to alanine of either of the conserved cysteines at HPV16 L2 amino acids 22 or 28, or proline 29, eliminated WW1 binding, as previously described for MAb RG1 [352]. However, while RG1 binding to HPV16 L2

was also previously reported to be affected if lysine 20 was mutated to alanine, WW1 binding was not affected by this mutation.

**S10 recognizes a conformational epitope at approximately the 63-89 L2 region of HPV 6, 11, 44 and 58**

Despite reactivity of S10 with HPV6 L2 polypeptide by ELISA and Western blot, S10 failed to bind to any of the overlapping 15-mer HPV6 L2 peptides in the array by ELISA (data not shown). Although we cannot rule out that a 5 residue overlap is insufficient to present all of the B cell epitopes within HPV6 L2, this discrepancy suggests the possibility that S10 recognizes a specific conformation of L2 that is absent from the peptide library, but can be presented by refolding of recombinant HPV6 L2 protein re-folding in situ on microtiter plates or PVDF membranes. To further confirm the genotype-specificity of S10, we performed ELISAs and Western blotting against HPV6, 11, 44, 58 and HPV16 pseudovirion preparations. The reactivities were consistent to data obtained with bacterially-expressed L2, in that S10 could react with L2 in pseudovirions of HPV6, 11, 44, and 58, but not HPV16, by both Western blot and ELISA (Figure 23A-B).

Since S10 binds within residues 12-88 of HPV6 L2 but not to the overlapping peptide array, an alternative strategy was devised to map its epitope. Specifically, a Western blot of three previously described L2 fusion proteins containing HPV6 L2 residues 12-88 (within L2  $\alpha$ 11-88 $\times$ 8 [344]), HPV6 L2 12-46 and 63-88 (within L2 $\alpha$ 11-88 $\times$ 8 $\Delta$ TM [7]), and 12-46 (within 13-47 $\times$ 15) [353]. S10 bound to  $\alpha$ 11-88 $\times$ 8 and  $\alpha$ 11-88 $\times$ 8 $\Delta$ TM but not the 13-47 $\times$ 15 fusion protein (Figure 23C). This indicates that the L2 63-89 region is required for S10 recognition of L2 and that a particular conformation may be important for this interaction.

### ***In vitro* neutralization of HPV PsV by WW1 and S10**

As Western blot and ELISA results are not indicative of functional neutralization activity, we next assessed using the L1-PBNA the neutralization capabilities of WW1, RG1 and S10 against HPV6/16/31/35/45/58 PsV serially titrated from 1666 $\mu$ M. An irrelevant rat IgG was also included as a negative control (Table 12). Consistent with previous findings [13], RG1 could only neutralize HPV16 and 18 (<13nM) [13, 68], whereas WW1 neutralized HPV16 (<13 nM), HPV18 (26nM) and HPV45 (69 nM). WW1 however did not neutralize HPV31 or 58 HPV PsV even at 1666nM, S10 also failed to consistently show any neutralization activity even against HPV6 PsV (Table 12).

We then performed passive transfer studies of WW1 and S10 followed by vaginal challenge of mice with pseudovirion of different HPV types, as it is a more sensitive and biologically relevant test than *in vitro* neutralization [18, 300]. The rat MAbs were administered intra-peritoneally to each naïve mice (n=5) in a single dose of 50 $\mu$ g. Given that 2mL is the estimated plasma volume for a mouse, this corresponds to a concentration of ~166nM assuming equal distribution and no degradation. These mice were then subjected to vaginal challenge with the same PsV types used in the L1-PBNA. Passive transfer of 50 $\mu$ g WW1 conferred significant protection against vaginal challenges with HPV16 (98.9%), HPV45 (97.2%), or HPV58 (93.7%) but not HPV6 or HPV31. Consistent with its *in vitro* neutralization data, passive transfer of 50 $\mu$ g RG1 protected mice against vaginal challenge with HPV16 PsV (100%), but not the other HPV types 31, 45 or 58. Surprisingly, in contrast to our L1-PBNA findings, mice that received 50 $\mu$ g of S10 also exhibited a reduction of luciferase activity (87.6%) after challenge with HPV6 PsV and some protection was observed against HPV 58 PsV (67.8%), although no significant protection was seen for S10 against HPV types 16, 31 and 45 (Table 12).

The inconsistency of *in vitro* and *in vivo* data assessing the inhibition by WW1 for HPV58 and S10 for HPV6 and HPV58 infectivity suggests that the L1-PBNA may be insufficiently sensitive for L2 neutralizing antibody analysis. However, use of the furin-cleaved neutralization assay (FC-PBNA) to re-analyze both WW1 and S10 provided *in vitro* neutralization data more consistent with the findings by passive transfer; WW1 neutralized in the nano-molar range all HPV types tested including HPV58 (2.08nM) except HPV6 and HPV31 (Table 12), whereas RG1 neutralized only HPV16 and HPV18. Importantly, using the FC-PBNA, we found that the IC<sub>50</sub> were ~10-fold lower than when measured using the L1-PBNA. Further, S10 could also neutralize HPV6 (8.44nM) and HPV58 (4.15nM) in the FC-PBNA, but this was not detected by L1-PBNA.

#### **Affinity of MAbs for HPV L2**

Despite WW1 recognizing epitopes within the same L2<sub>17-36</sub> region for several HPV types as RG1, there were observed differences between Western blot and ELISA reactivity as well as neutralization towards different HPV types. For example, WW1 is reactive to HPV31 via Western blot or ELISA, however, WW1 is non-neutralizing to HPV31. To further investigate and understand if this is related to binding kinetics for each specific virus type, we performed quantitative measurement of antibody affinity using the BLItz® Label-free assay system (ForteBio Inc.) using streptavidin biosensors loaded with biotinylated HPV L2<sub>17-36</sub> peptides from HPV 6/16/31/45 and 58 respectively. Following loading, the L2 sensors were exposed to different concentrations of RG1 and WW1 IgG to measure the association rate (K<sub>a</sub>), and dissociation rate (K<sub>d</sub>) to calculate the equilibrium dissociation constant (K<sub>D</sub>) (Table 13). The K<sub>D</sub> of RG1 towards HPV16 L2 peptide was 8nM while the K<sub>D</sub> values were 10-100 fold higher for HPV types 6/31/45/58. These findings corresponded with the IC<sub>50</sub> determined by the L1-PBNA, <13nM for HPV16 and >1666nM for the remaining types tested. Likewise, RG1 gave robust protection against vaginal challenge with HPV16 upon passive transfer at an estimated concentration of 166nM, but it was not protective against challenge with HPV types 6/31/45/58.

In contrast, the  $K_D$  values for WW1 were consistently one log higher than RG1. For example, the WW1  $K_D$  value for HPV16 was 37nM ( $K_D=10^{-8}$  M range), consistent with the *in vitro* neutralization molarities observed in the FC-PBNA for WW1 against HPV16. The  $K_D$  values for WW1 against HPV31/45/58 were 261nM, 277nM and 409nM respectively ( $K_D=10^{-7}$  M range) also (Table 13) and there was no clear correlation between WW1 affinity and *in vitro* neutralization. Thus WW1 has a lower affinity for this peptide compared to RG1 but this could also account for its broader western and ELISA reactivity. Following the characterization of WW1 and S10, we next proceeded to use WW1, due to its broadest reactivity and neutralization capabilities, to further understand how L2-neutralization occurs.

### **FcRn is not required for protection via passive transfer of WW1**

It is not well understood how neutralizing IgG in serum protect against a viral inoculum locally administered in the vaginal lumen since virions do not cross the basement membrane. Studies indicate that IgG concentrations in vaginal fluid exceeds that of IgA, reflecting transcytosis of IgG from the plasma into the genital tract via Fc interaction with the neonatal Fc Receptor (FcRn) [354]. FcRn-mediated intracellular neutralization has been described in polarized epithelial cells [355]. Therefore, to assess if FcRn is required to transport protective antibodies into the genital tract to prevent HPV infection, we performed the passive transfer of WW1 IgG followed by HPV16 vaginal challenge in both wild type and FcRn deficient C57BL/6 mice. Both groups were equally susceptible to infection after pre-incubation with nonoxynol-9 (N9) and vaginal challenge with HPV16 PsV (Figure 23A). Unexpectedly, upon systemic administration of WW1 to groups of ten mice followed by vaginal challenge with HPV16 PsV, similar protection was observed wild type and FcRn deficient mice (Figure 23B), suggesting that transcytosis is not required or possibly, although unlikely, that there is another undefined transcytosis mechanism that does not depend on FcRN,



A more logical alternative is that the IgG might reach the virus via exudation since HPV infection in the genital tract of mice requires epithelial trauma. The epithelial trauma is elicited in mice by brushing or administration of nonoxynol-9 in the vagina, presumably to allow the virions to reach the basement membrane and access basal keratinocytes. Since active transport of IgG into the vaginal lumen by FcRn was not required for protection, we examined whether exudation at the site of challenge allowed systemic WW1 IgG to enter the genital tract. Hence, 6 hours after systemic administration of WW1 IgG, three groups (n=5) of Balb/c mice were administered N-9 (50 $\mu$ L; 4% v/v) or cytobrush treatment in the vaginal lumen or no additional treatment. Twelve hours later, vaginal washes with saline were then collected and tested for WW1 IgG. The level of WW1 detected in the vaginal lavage was dramatically increased by N9 treatment (mean=51.4 nM) compared to the no treatment control (mean=2.4 nM) (Figure 24D). Surprisingly, the cytobrush method exhibited a less dramatic increase in WW1 and was approximately 5-fold lower in concentration compared to mice with N9 treatment (mean=8.6 nM) (Figure 24D). Together these results suggest that FcRn is not required for the transport of WW1 into the mouse genital tract since epithelial trauma required for HPV infection permits exudation of plasma IgG to meet the inoculum.

### **WW1 (Fab')<sub>2</sub> fragment is less protective against HPV16 challenge than whole IgG**

To further dissect how L2 antibody neutralization occurs, we decided to examine the importance of the Fc portion of L2 antibodies in mediating protection. WW1 (Fab')<sub>2</sub> fragments were prepared by digestion on pepsin-Sepharose beads, and undigested Fc removed by passage through protein G-Sepharose. Completeness of digestion and purity of the fragments was assessed by reducing and non-reducing SDS-PAGE and Coomassie staining (data not shown). To determine whether the (Fab')<sub>2</sub> fragments retained epitope binding, their recognition of either HPV16 PsV or synthetic HPV16 L2 17-36 peptide was first examined by ELISA using peroxidase-linked mouse MAb specific for rat kappa light chain as the secondary antibody. Both WW1 IgG and WW1

(Fab')<sub>2</sub> fragments were able to bind to both HPV16 L2 peptide and HPV16 PsV while S10, as expected, did not (Figure 25A). When peroxidase-linked mouse anti-rat IgG2a Fc was used as the secondary antibody, no reactivity was detected with the (Fab')<sub>2</sub> preparation, suggesting the (Fab')<sub>2</sub> preparation was free of whole WW1 IgG2a. Conversely whole WW1 IgG2a was detected with mouse anti-rat IgG2a Fc (Figure 25B). Taken together, the results show that WW1 binding affinity for HPV16 was Fc-receptor independent.

To address the relevance of the Fc to protection, naïve mice were passively transferred with titrations of WW1 whole IgG or its (Fab')<sub>2</sub> fragment and then 6 h later challenged with HPV16 PsV. Whole WW1 IgG provided robust protection (94%) at 7.5µg (25nM), while the administration of WW1 (Fab')<sub>2</sub> failed to achieve >90% protection even at 15µg (75nM) (Figure 25D). This difference might reflect lower stability of the (Fab')<sub>2</sub> *in vivo* as compared to the IgG, or the loss of Fc-mediated effector functions.

### **Contribution of phagocytes to WW1-mediated protection**

Opsonization via recognition of the Fc of IgG bound to extracellular pathogens promotes their engulfment and clearance by phagocytes. Since WW1 F(ab')<sub>2</sub> lack the Fc portion, it is possible that their lower protective capacity *in vivo* reflects an inability to opsonize extracellular HPV16 virions. Indeed, it was also previously described that neutrophils accumulated at the site of HPV16 PsV challenge in mice treated with L2 antibody and was associated with disappearance of virions over 18h [294]. However, the role of phagocytes in L2 antibody mediated protection was not directly tested. Therefore, we compared the ability of passively transfer of WW1 IgG to protect against vaginal challenge with HPV16 in normal mice or those depleted of neutrophils and GR1+ macrophages (Figure 28). While the depletion of neutrophils and GR1+ macrophages had no impact on HPV16 PsV infection in the genital tract (Figure 26D), passive transfer of WW1 IgG (25µg/mouse, ~83nM) was significantly less protective (P<0.05) against HPV16 in

mice depleted of neutrophils and GR1+ macrophages (~82%) as compared to control mice (~95%) (Figure 26F). This implies that the Fc portion of WW1 IgG contributes to protection by promoting phagocytosis of extracellular virus, as suggested by prior immunofluorescent studies [294].

### **Fc of WW1 IgG also promotes viral neutralization *in vitro***

The HPV16 neutralization potentials of WW1 IgG and (Fab')<sub>2</sub> were also compared *in vitro*. Since opsonization should only be relevant *in vivo* as phagocytes are absent, and that ELISA binding affinity was similar for both whole IgG and (Fab')<sub>2</sub> (Figure 25A-B), it was expected that no difference should be observed. Unexpectedly, the IC<sub>50</sub> values for WW1 IgG and (Fab')<sub>2</sub> were 26nM and 292nM respectively (Figure 25C) suggesting that WW1 IgG is ~10-fold more potent in neutralizing than WW1 (Fab')<sub>2</sub>. This difference in neutralization is unlikely to reflect Fc-mediated complement activation since the 293TT cells were cultured in heat inactivated serum. Interestingly, more complete neutralization of HPV16 could be achieved *in vitro* by WW1 (Fab')<sub>2</sub>, than protection was observed in the mouse challenge model if saturating amounts of WW1 (Fab')<sub>2</sub> were utilized. This difference between neutralization *in vitro* and *in vivo* despite using saturated quantities of WW1 (Fab')<sub>2</sub> may reflect lower stability of the (Fab')<sub>2</sub> than IgG *in vivo* as well as impaired Fc-mediated effector function. Nevertheless, our findings suggests that the Fc of WW1 contributes to protection both by opsonization of extracellular virus, and enhancing neutralization *in vitro*.

### **WW1 does not utilize TRIM21-dependent intracellular neutralization**

Recently, a mechanism termed Antibody-Dependent Intracellular Neutralization (ADIN) was described [350] in which the cytosolic Fc receptor TRIM21 binds to a conserved region (H433, N444, H435) within the Fc portion of antibody bound to virions that enter the cytosol, shuttling the complex to the proteasome for rapid degradation [283, 331]. As L2 may carry the viral DNA to nucleus via the cytosol, we decided to investigate if the lower neutralization capabilities of

WW1 F(ab')<sub>2</sub> *in vitro* reflected loss of TRIM21-dependent ADIN in the cytosol because of the Fc removal. First we compared the capacity of WW1 to neutralize HPV16 infection of wild type and TRM21-deficient mouse embryonic fibroblasts. HPV16 infectivity was similar in both cell lines (data not shown) although requiring more virus compared to 293TT or LoVoT cell lines. Importantly, there was also no difference in neutralization titer when a variety of serum and antibodies were measured in either TRM21-deficient or wild type mouse fibroblasts (Figure 26A). However, the failure to see a difference might reflect the inability of the Fc of the rat WW1 IgG to bind appropriately with mouse TRIM21 to effect ADIN.

To circumvent potential interaction problems due to this species difference, we next synthesized a hybrid cDNA fusing the variable regions of WW1 onto a human IgG1 Fc and subcloned it into a mammalian expression vector and termed the antibody: JWW1. In addition, we created a second construct (JWW1-A3) in which the three critical residues of the TRIM21 binding region of human IgG1 Fc were all mutated to alanine (H433A,N434A, H435A), a change previously shown to eliminate TRM21 binding [356]. Both antibodies were produced by transfection of 293 cells under serum-free conditions, and purified from clarified culture supernatants using protein G columns. Subsequently, both the JWW1 and the JWW-A3 antibodies were tested for binding efficacy against HPV16 L2 (Figure 26B) and for *in vitro* neutralization of HPV16 PsV (Figure 26C). No difference in titer was seen in either assay. These findings suggest that while the Fc portion of WW1 does contribute to HPV16 neutralization *in vitro*, this does not involve TRIM21-mediated ADIN.

## **DISCUSSION**

Since the neutralizing MAb RG1, which was produced by vaccination with full length HPV16 L2, is cross-reactive to only a small subset of oncogenic HPV types, we hypothesized that a multitype L2 immunogen, such as L2 $\alpha$ 11-88x8 which contains the L2 aa11-88 regions of 8

different HPV genotypes, would yield more broadly reactive MAbs. This approach we generated the narrowly cross-neutralizing S10 and the more broadly cross-reactive WW1 MAb. WW1's greater breadth of cross-reactivity compared to RG-1 (despite both recognizing epitopes within L2 17-36 aa) is consistent with the broader neutralizing antibody response elicited by the multimer as compared with a single type L2 immunogen, and may further reflect superior cross-linking of broadly reactive B cell receptors by the multimer because the chain of constituent subunits are derived from different genotypes.

Prior descriptions of L2-specific neutralizing epitopes suggest that they are linear [6]. However, the neutralizing MAb S10 recognizes an C-epitope with conformational dependence in the amino terminus of L2 that is also displayed by the immunogen, L2  $\alpha$ 11-88x8. Indeed, improved Western blotting results were obtained on extended incubation, suggestive of refolding. Because of this conformational reactivity, we were unable to fully define the epitope recognized by S10 using a peptide library, although it was partially mapped to within residues 68-89 of L2 of predominantly low risk HPV genotypes (Figure 23). Thus, we concentrated our mapping efforts upon WW1 because of its much broader binding to hrHPV, recognition of short peptides and greater neutralizing capacity.

The cross-reactive natures of WW1 and RG1 relate to their recognition of the only two cysteine residues in L2, (residues 22 and 28), both conserved in all HPV types, as well as the conserved proline 29. ClustalW analysis of the 18-32 region (Figure 21) suggests that RG1 requires K at residue 20 for robust binding, whereas WW1 is tolerant to Q/R/K/S at this position and thus has a broader spectrum of binding. Although further direct testing with peptides is required, this ClustalW comparison also suggests that WW1 tolerates A or S at L2 residue 25 as well as T or S at residue 21.

We and others previously observed that the conventional *in vitro* pseudovirion-based neutralization assay (L1-PBNA) was insensitive towards L2-mediated neutralizing antibodies as compared to either passive transfer studies or when utilizing furin-cleaved pseudovirion-based neutralization assays (FC-PBNA) [11, 13, 331]. Here, S10 did not detectably neutralize HPV6 in the L1-PBNA even at a concentration of 1666nM, but was 88% protective against HPV6 challenge upon passive transfer of 50µg S10 (~166nM *in vivo*), and had an IC<sub>50</sub> of 7nM using the FC-PBNA (Table 12). Likewise, neither WW1 nor S10 detectably neutralized HPV58 in the L1-PBNA even at a concentration of 1666nM, yet passive transfer studies of these antibodies (50µg/mouse, ~166nM) protected mice, and both S10 and WW1 were detectably neutralized HPV58 the FC-PBNA (Table 12) [13]. Thus, the FC-PBNA again better correlates with the HPV PsV murine passive transfer model [18], which is presumably the most biologically relevant assay, and therefore should be considered for high throughput *in vitro* measurement of serum neutralizing antibody responses to L2-based vaccination.

While WW1 and RG1 are cross-neutralizing (Table 12), IC<sub>50</sub> values differed substantially by HPV type. For example, the IC<sub>50</sub> of WW1 for HPV16 was 0.52nM but higher for HPV45 (4.51nM) or HPV58 (6.71nM) (FC-PBNA, Table 1) presumably reflecting the impact of variations in the epitope sequence on the binding affinity. Higher binding affinity to peptide determined by interferometry was associated with the strongest protection in the passive transfer model. However, the K<sub>D</sub> measurements for MAb binding to L2 peptide were typically >10-fold higher compared to their IC<sub>50</sub> neutralization values in the FC-PBNA, but more consistent with the L1-PBNA. This implies that the WW1 may be binding bivalently in the FC-PBNA but not the L1-PBNA. We attempted to verify this by preparing Fab' fragments of WW1 by papain digestion. However, while the proteolysis was successful, the resulting fragments were not reactive with L2 by ELISA (data not shown). Interestingly the IC<sub>50</sub> in the WW1 passive transfer study was more consistent with the FC-PBNA rather than the higher values observed using the conventional 'L1-

PBNA'. This suggests that bivalent binding of L2 by neutralizing antibodies may also occur in vivo. We propose that the lower sensitivity of the L1-PBNA for L2-specific WW1, but not L1-specific neutralizing antibodies, reflects monovalent binding of WW1 in this assay, i.e. more efficient furin cleavage of virions in the FC-PBNA (or in vivo) better permits bivalent binding of L2-specific neutralizing antibodies, but has no effect upon the L1-specific antibody detection. Conversely partial furin cleavage of L2 in the L1-PBNA is sufficient to permit infection, but only monovalent binding by L2-specific neutralizing antibodies, and their IC50 measured in this assay is higher than for the FC-PBNA or passive transfer experiments.

While HPV L1 VLP vaccines elicit high titers of neutralizing serum antibodies and are remarkably effective for prophylaxis, they lack therapeutic efficacy. This suggests that they provide sterilizing immunity via neutralizing antibody. However, it is still unclear how neutralizing serum IgG reaches the inoculum at the site infection in genital epithelia, since HPV does not cross the basement membrane. Significant quantities of IgG are present in vaginal fluid and the ratio to IgA varies across the menstrual cycle [357]. In mice, IgG are brought into the genital tract via Fc-binding to FcRn [354]. After passive transfer into FcRn deficient mice there was minimal passive diffusion of WW1 into the vagina (Figure 24C), but physical or chemically-induced epithelial trauma, required for effective viral challenge, greatly enhanced its transit (Figure 24C). Surprisingly, we found that N9 treatment releases more antibody into the vaginal vault compared to the physical microtrauma elicited by brushing (Figure 24C). This suggests that N9 (a mild detergent and widely used spermicide) can cause significant disruption to the epithelial barrier, although the BM remains intact.

Importantly, there was no difference in protection against HPV16 challenge between wildtype and FcRn deficient mice after passive transfer of WW1 IgG (Figure 24B). This suggests that active FcRn-mediated transcytosis of systemically administered WW1 IgG is not required to

protect mice from vaginal challenge with HPV, but rather passive leakage of WW1 IgG at the site of wounding (i.e. exudation) is likely sufficient to mediate protection (Figure 24C). However, FcRn likely contributes other functions such as extending antibody half-life while in the systemic circulation [358, 359]. Our findings suggest that in HPV vaccinated patients the neutralizing serum antibodies may reach the viral inoculum at the genital mucosa as a result of micro-trauma to the epithelium during sexual intercourse and local exudation. This notion is consistent with robust protection observed across all stages of the menstrual cycle despite fluctuating IgG levels in vaginal fluid [312, 360]. However a limitation of passive transfer as a model for active vaccination is that it does not account for the possibility of B lymphocytes might be present within the cervicovaginal epithelium and producing neutralizing antibody locally.

Previous studies found that L2-specific neutralization does not prevent HPV interaction with its primary receptor, HSPG, on the basement membrane during infection, but the antibody-bound virions fail to bind basal keratinocytes and are lost over time [339]. The disappearance of the antibody-bound virions was associated with cellular infiltrates consisting mainly of neutrophils, suggestive of opsonization and phagocytosis of the L2 antibody-bound virion upon Fc-dependent recognition by the neutrophils and/or wound macrophages. Here we observed that, while binding of WW1 to virions was Fc-independent (Figure 25A-B), overall protection *in vivo* by WW1 (Fab')<sub>2</sub> was weaker than for the IgG (Figure 25D) suggesting that the Fc does contribute to the potency of protection.

To examine whether opsonization contributed to protection, we performed WW1 IgG passive transfer experiments in animals pre-treated with monoclonal antibody RB6-8C5 which specifically depletes neutrophils as well as macrophages at the site of wounding [361]. While depletion of these phagocytes does not affect HPV16 PsV infection (Figure 26E), WW1-mediated protection was reduced ~10-fold (Figure 26F). These findings correlate with the WW1 (Fab')<sub>2</sub>



passive transfer experiments suggesting a secondary mode of L2-antibody mediated protection is via phagocytosis by infiltrating Ly6G/C+ neutrophils/macrophages, likely attracted by the epithelial trauma. In addition, antibodies that bind to virions but fail to neutralize *in vitro* may still contribute to protection *in vivo* via opsonization.

Surprisingly, WW1 (Fab')<sub>2</sub> is also ~10-fold less neutralizing than IgG *in vitro*, suggesting that the Fc region is important for a second mode of viral inactivation. However, this is not the recently described TRIM21-dependent ADIN (Figure 26A-C). A post-uptake mechanism of neutralization has recently been suggested for L2-specific antibody by preventing exit of virions from vesicles to gain access to the cytosol/nucleus [84]. This mechanism is consistent with our findings that ADIN is irrelevant for WW1-mediated neutralization since TRIM21 is a cytosolic protein whereas the antibody is proposed to trap the virion within the endosome. In light of this, we speculate that the Fc might contribute to neutralization by enhancing the trafficking of antibody-bound virions to the lysosomes for degradation since it has been documented that the bulk of non-infectious particles accumulate in LAMP1+ vesicles after infection. Finally, *in vivo* despite saturating levels of WW1 (Fab')<sub>2</sub> (75nM), complete protection was never achieved, and we speculate that this reflects in part a shorter half life *in vivo* as compared to IgG because of the role of the Fc in recovery in the kidney (Figure 25D).

In summary, we have created two new broadly reactive L2-specific MAbs and shown that L2 antibody mediates protection both by opsonization of extracellular virions and direct neutralization. The previously unknown role of opsonization in protection may help account for why L2 antibodies are more active in the HPV PsV mouse challenge model as compared to *in vitro* neutralization experiments. Additional study is needed to define the mechanisms of post-uptake neutralization, but the findings herein support the potential of L2 vaccination for the broad prevention of genital HPV infection.

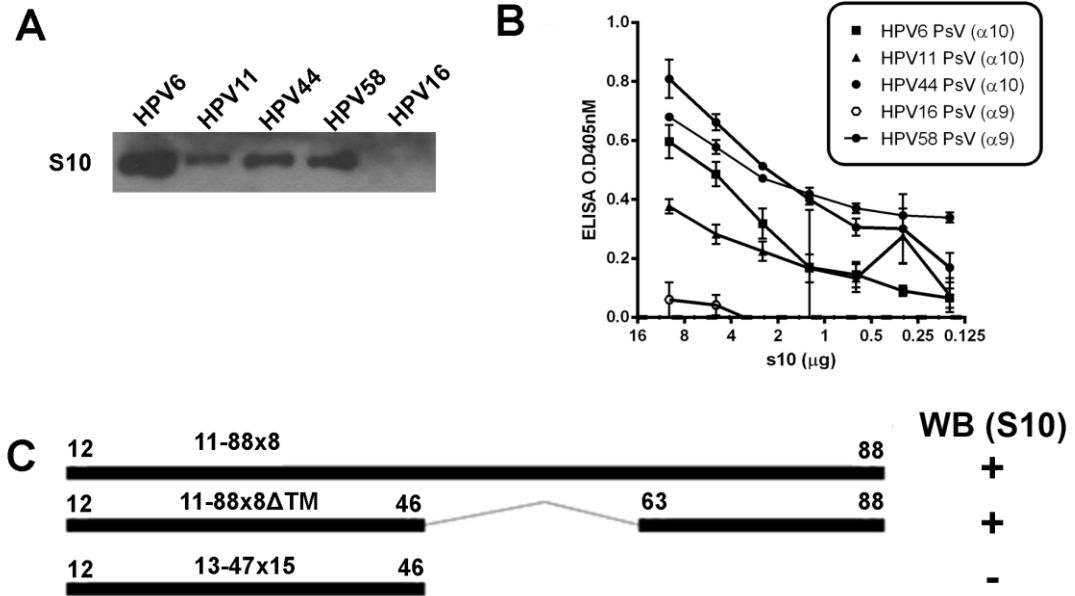
Sub-family	HPV type	Mucosal/ Cutaneous	HPV16 L2 (18-32) region	Antibody Reactivity		
				RG-1 (17-36)	WW1 (18-32)	L2α(11- 88)X8 Rabbit Sera
α1	32	M	LYQTCKASGTCPPDV	Red	Green	Green
	42	M	LYQTCKASGTCPPDV	Red	Green	Green
α2	3	C/M	LYRTCKAAGTCPPDV	Red	Green	Green
α5	26	M	LYKTCKAAGTCPPDV	Green	Green	Green
	51	M	LYSTCKAAGTCPPDV	Red	Green	Green
α6	53	M	LYQTCKQSGTCEPDV	Red	Green	Green
	56	M	LYKTCKLSTGTCPEVDV	Green	Green	Green
	66	M	LYKTCKLSTGTCPEVDV	Green	Green	Green
α7	18	M	LYKTCKQSGTCPPDV	Green	Green	Green
	39	M	LYRTCKQSGTCPPDV	Red	Green	Green
	45	M	LYRTCKQSGTCPPDV	Red	Green	Green
	59	M	LYKTCKQAGTCTPSDV	Green	Green	Green
	68	M	LYKTCKQSGTCTPSDV	Green	Green	Green
	70	M	IYKTCKQSGTCPPDV	Green	Green	Green
α8	40	M/C	LYQTCKAAGTCPPDV	Red	Green	Green
	43	M/C	LYQTCKAAGTCTPSDV	Red	Green	Green
α9	16	M	LYKTCKQAGTCTPPDI	Green	Green	Green
	31	M	LYQTCKAAGTCTPSDV	Red	Green	Green
	33	M	LYQTCKAAGTCTPPDV	Red	Red	Green
	35	M	LYRTCKAAGTCPPDV	Green	Green	Green
	52	M	LYQTCKASGTCPPDV	Red	Green	Green
	58	M	LYQTCKASGTCPPDV	Green	Green	Green
α10	6	M	LYQTCKLSTGTCPPDV	Red	Red	Green
	11	M	LYQTCKLSTGTCPPDV	Red	Red	Green
	44	M	LYQTCKAAGTCTPSDI	Red	Green	Green
α11	34	M	LYKTCKQSGTCTPPDI	Green	Green	Green
	73	M	LYKTCKQAGTCTPPDV	Green	Green	Green
β1	5	C	IYQTCKQAGTCTPPDV	Green	Green	Green
	8	C	IYQTCKQAGTCTPPDV	Green	Green	Green
β2	38	C	IYRGCCKASNTCTPPDV	Red	Red	Green
	23	C	IYKGCCKASGTCPPDV	Red	Red	Green
β3	76	C	IYQSCCKAAGTCTPPDV	Red	Green	Green

Figure 21. Summary of RG1 and WW1 western blot reactivity towards 34 clinical relevant HPV types from the alpha and beta sub families. Red boxes indicate no rMAb binding (negative Western blot result) and green boxes indicates rMAb binding occurred (positive

Western blot result). The WW1 epitope on HPV16L2<sub>18-32</sub> and corresponding regions on other HPV types was aligned using the UCSF chimera software. Red boxes in the multiple sequence alignment indicate putative key amino acid mutations from HPV16L2<sub>18-32</sub> that resulted in WW1 non-reactivity.

HPV type		Antibody Reactivity	
		RG-1 (17-36)	WW1 (18-32)
16 L2 WT	Q L Y K T C K Q A G T C P P D	+	+
16 L2 K20A	Q L Y A T C K Q A G T C P P D	-	+
16 L2 C22A	Q L Y K T A K Q A G T C P P D	-	-
16 L2 C22S	Q L Y K T S K Q A G T C P P D	-	-
16 L2 C28A	Q L Y K T C K Q A G T A P P D	-	-
16 L2 C28S	Q L Y K T C K Q A G T S P P D	-	-
16 L2 P29A	Q L Y K T C K Q A G T C A P D	+/-	+/-

Figure 22. **Conserved cysteines in L2 are required for WW1 binding.** Analysis of RG1 and WW1 binding with either wildtype HPV16 PsV or point mutant HPV16 PsV at the HPV16L2<sub>18-32</sub> site. The WW1 epitope on HPV16 and corresponding point mutant L2 regions on other mutants was aligned using the UCSF chimera software. ‘+’ indicates strong binding, ‘+/-’ indicates weak binding and ‘-’ indicates no binding.



**Figure 23. S10 recognizes a conformational epitope.** Western blot (A) and ELISA (B) reactivity of MAb S10 with L2 of HPV6, 11 and 44 ( $\alpha$ 10) and the two distantly related  $\alpha$ 9-types, HPV16 and 58. Western blot analysis against three different L2 fusion proteins to map S10's epitope (C), '+' indicates positive western blot result, '-' indicates negative result.

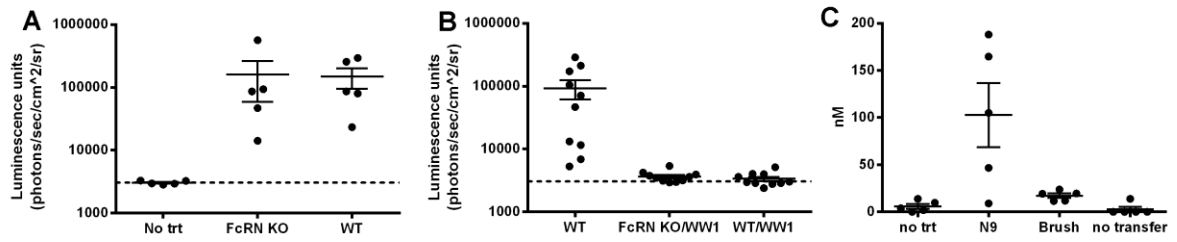


Figure 24. **FcRn-independent release of WW1 into the vagina.** (A) HPV16 PsV encoding a luciferase reporter plasmid infects FcRn-KO and wildtype (WT) C57/BL6 mice equivalently after vaginal challenge. (B) Passive transfer of WW1 (50 $\mu$ g) protects both FcRn-KO or WT mice against vaginal challenge with HPV16 PsV (B). Higher concentrations of WW1 IgG are present in vaginal lavage samples obtained from WT and FcRn-KO mice after N9 treatment as compared to cytobrush induced microtrauma of the murine cervicovaginal epithelium (C).

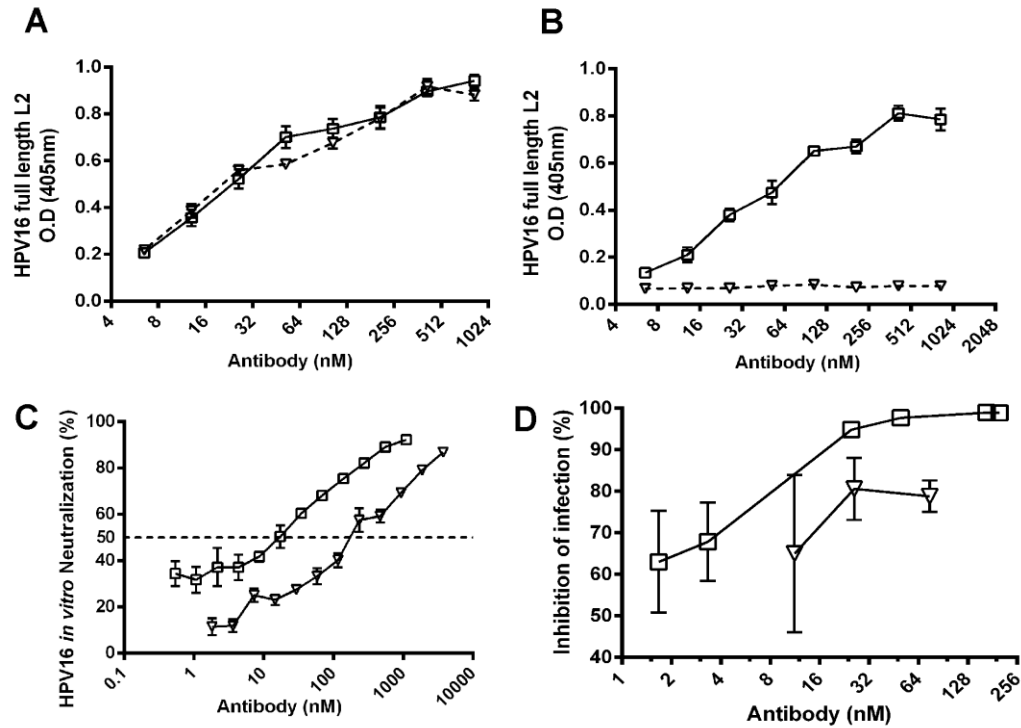


Figure 25. **WW1 IgG neutralizes HPV16 better than its F(ab')<sub>2</sub>**. WW1 whole IgG and its F(ab')<sub>2</sub> bind comparably to HPV16 L2 protein by ELISA when using an Fab-specific secondary antibody (A). The WW1 Fab<sub>2</sub> preparation lacked detectable whole WW1 IgG as anti-IgG2a secondary antibody failed to detectably react in an HPV16 L2 ELISA (B). Whole WW1 IgG2a can neutralize HPV16 better *in vitro* in the FC-PBNA than its F(ab')<sub>2</sub> (C), and likewise protects more strongly *in vivo* upon passive transfer into naïve mice and vaginal challenge with HPV16 (D) despite similar binding to HPV16 L2 when measured by ELISA (A).

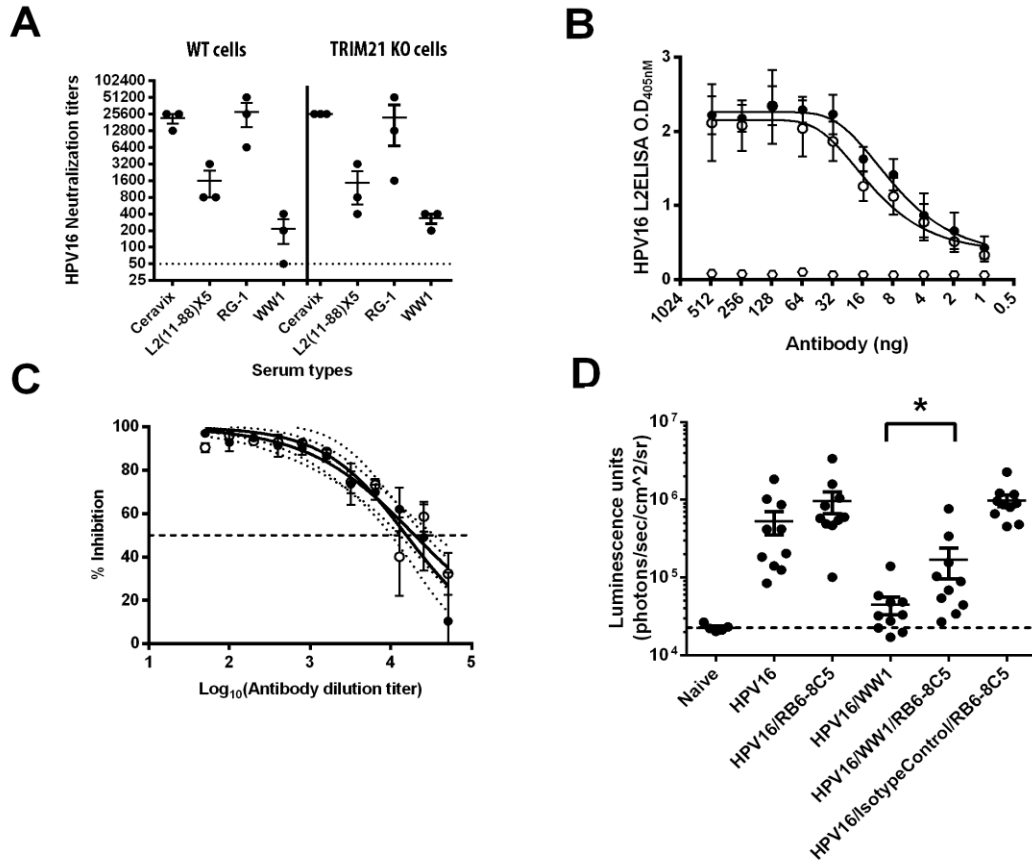


Figure 26. **Fc of WW1 promotes opsonization but not TRIM21-mediated intracellular neutralization.** (A) Measurement of in vitro neutralizing antibody titer in the L1-PBNA for mouse antisera to Cervarix or L2 $\alpha$ 11-88x5, or MAbs WW1 and RG-1. The neutralization assays tested the IC<sub>50</sub> for HPV16 infection of either wildtype MEF cells (left) or TRIM21KO MEF cells (right). (B) HPV16 L2 ELISA and neutralization assay (C) of humanized WW1 (closed circles) and humanized WW1 with TRIM21 binding site mutations (open circles) upon infection of LoVoT cells with HPV16 pseudovirions. (D) Passive transfer of WW1 IgG into wildtype or neutrophil/macrophage depleted mice (n=10) followed by HPV16 pseudovirus challenge.



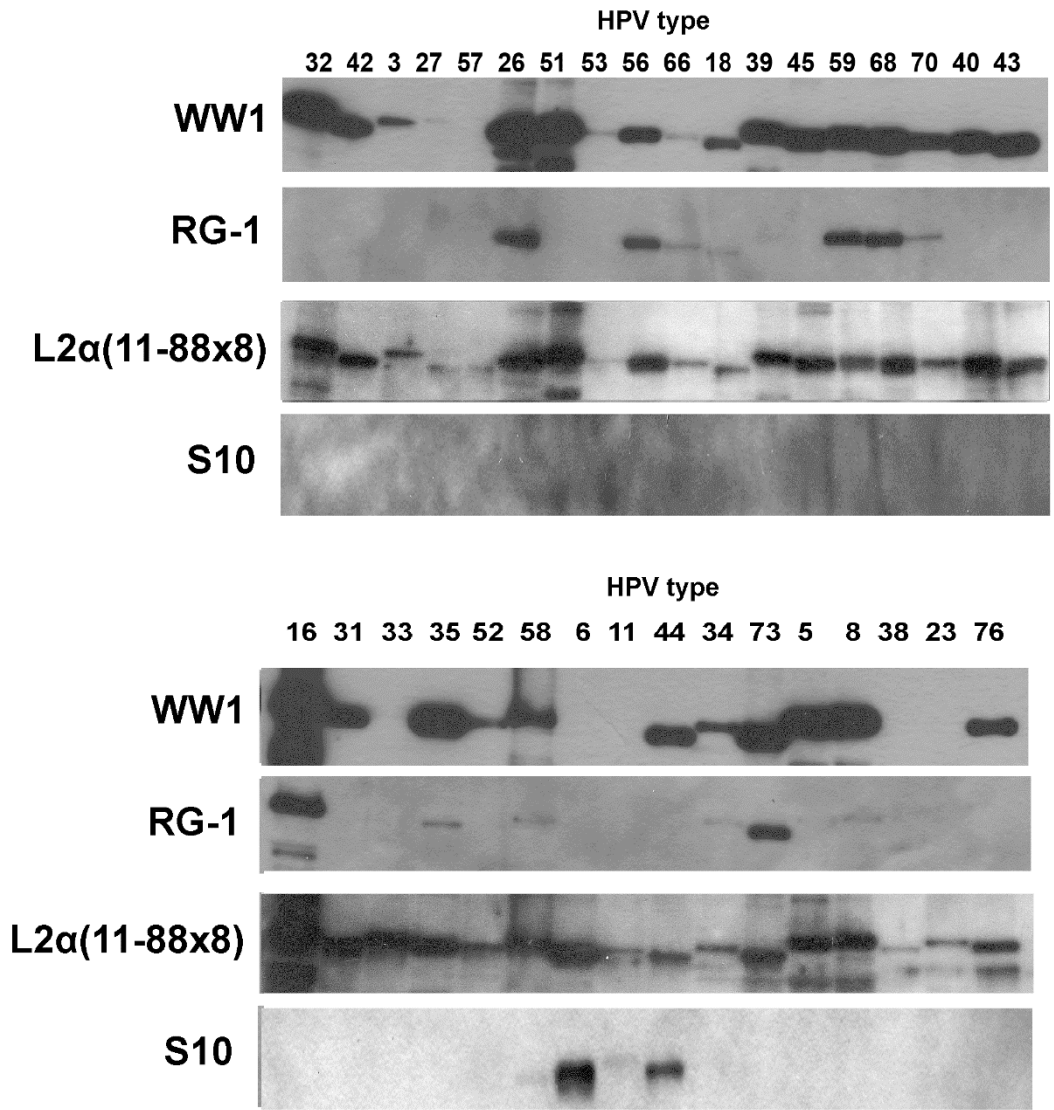


Figure 27. **Western blot analysis of pseudovirion preparations of 34 clinically relevant HPV types.** Reactivity was assessed for RG1, WW1, S10 and, as loading control, rabbit antiserum to L2α(11-88)x8.

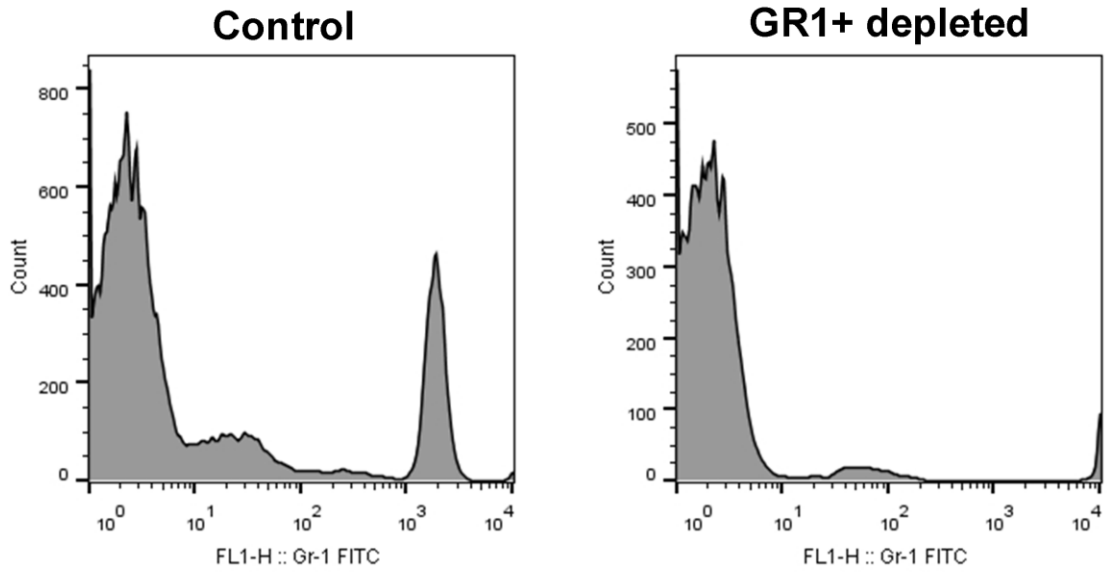


Figure 28. Depletion of GR1+ neutrophils and macrophages in Balb/c mice upon pre-treatment with MAb RB6-8C5.

**Table 12. Neutralization and protection by MAbs against different HPV genotypes.**

Summary of neutralization IC<sub>50</sub> (nM) against HPV 6/16/18/31/45 and 58 for MAb RG1, WW1, and S10 determined by 2-fold antibody titration from 1666nM and measured using the L1-PBNA or FC-PBNA. Percent protection obtained by passive transfer of 50µg (166nM) MAb prior to challenge with HPV 6/16/18/31/45 and 58. N.D. not done.

<b>Genotype</b>	<b>Monoclonal Antibody</b>	<b>L1-PBNA IC<sub>50</sub> (nM)</b>	<b>FC-PBNA IC<sub>50</sub> (nM)</b>	<b>Protection by Passive Transfer (mean % in 5 mice)</b>
<b>HPV 6 (Luciferase Reporter)</b>	RG1	>1666	>1666	0.00%
	WW1	>1666	>1666	0.00%
	S10	>1666	8.44	87.6%
	Rat IgG control	>1666	>1666	0.00%
<b>HPV 16 (Luciferase Reporter)</b>	RG1	<13	0.26	100%
	WW1	<13	0.52	98.9%
	S10	>1666	>1666	0.00%
	Rat IgG control	>1666	>1666	0.00%
<b>HPV 18 (Luciferase Reporter)</b>	RG1	<13	0.28	N.D
	WW1	26	0.42	N.D
	S10	>1666	>1666	N.D
	Rat IgG control	>1666	>1666	N.D
<b>HPV 31 (Luciferase Reporter)</b>	RG1	>1666	>1666	0.00%
	WW1	>1666	>1666	0.00%
	S10	>1666	>1666	0.00%
	Rat IgG control	>1666	>1666	0.00%
<b>HPV 45 (Luciferase Reporter)</b>	RG1	>1666	>1666	0.00%
	WW1	69	4.15	97.2%
	S10	>1666	>1666	0.00%
	Rat IgG control	>1666	>1666	0.00%
<b>HPV 58 (Luciferase Reporter)</b>	RG1	>1666	>1666	0.00%
	WW1	>1666	2.08	93.7%
	S10	>1666	4.15	67.8%
	Rat IgG control	>1666	>1666	0.00%

**Table 13. Measurement of MAb affinity for L2 of different HPV by interferometry.**

Measurement of the dissociation constant for MAb RG1 and WW1 binding to the 17-36 amino

acid peptides of several HPV L2 was determined by interferometry using the BLItz system. S10

was unreactive to all peptides.

HPV L2 17-36aa	RG1			WW1		
	KD	Ka	Kd	KD	Ka	Kd
HPV 6	3.82E-07	1.94E+04	7.42E-03	1.79E-06	4.88E+03	8.71E-03
HPV 16	8.34E-09	7.92E+04	6.60E-04	3.70E-08	8.87E+03	3.28E-04
HPV 31	2.79E-07	2.50E+04	6.97E-03	2.61E-07	7.96E+03	2.08E-03
HPV 45	3.54E-07	2.48E+04	8.77E-03	2.77E-07	2.77E+03	7.68E-04
HPV 58	1.80E-07	3.69E+04	6.64E-03	4.09E-07	6.54E+03	2.67E-03

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## **V. Immunologic control of Mouse Papillomavirus 1**

### **Abstract**

Immune-suppressed patients, by genetics, drugs, or viral infection, display Human papillomavirus (HPV)-associated diseases at a higher frequency than immune-competent individuals who clear HPV infection without apparent disease thus suggesting HPV infection is controlled efficiently by an intact immune system. We show this in parallel via infection of mouse papillomavirus (MusPV1/MmuPV1) into C57/BL6, Balb/C or an out-bred immune-competent SKH-1 mouse model. Results indicate both genetic differences and altered immune statuses can result in sub-clinical infections which are readily reactivated upon stronger immune-suppression, explaining the varied capacity in disease rejection. Rejection of infection and disease requires cellular-mediated responses. In C57/BL6 mice, these immunodominant CD8 T-cell epitopes were mapped to MusPV E6 & -E7. A MusPV1 E6-specific effector CD8 T-cell line was generated and upon systemic administration, was able to traffic to the site of MusPV1 infection/disease and effect papilloma clearance, highlighting the promise of immunotherapy against papillomavirus infection and disease.

### **Introduction**

Papillomaviruses (PVs) are small, non-enveloped, double-stranded DNA viruses that produce papilloma/warts in a wide variety of organisms, although with strict host tropism [362, 363]. Over 120 human papillomaviruses (HPV) have been fully characterized. HPV are trophic for either mucosal or cutaneous epithelia [1, 337]. The mucosal HPV genotypes comprise mainly the  $\alpha$  species which are further categorized by oncogenicity; ‘low risk’ (lrHPV) types are associated with benign genital warts, whereas the ~15 ‘high risk’ (hrHPV) types have malignant potential [140]. Indeed, hrHPV are present in >99% of cervical cancers and are considered a necessary cause [139, 141]. The hrHPV, predominantly HPV16, also cause a subset of cancers at anogenital sites and the oropharynx [364]. In contrast, there are numerous cutaneous HPVs that can cause

benign papilloma on the skin such as common, plantar and flat warts. The types that are responsible include  $\alpha$  species (e.g. HPV 2, 27 & 57) as well as  $\gamma$  species (e.g. HPV4, 65),  $\mu$  species (e.g. HPV1) and the more studied  $\beta$  species families [1]. Cutaneous papillomavirus infection is near ubiquitous especially in school children [365] but most infections are not clinically apparent or spontaneously resolve [366, 367]. The  $\beta$  species types HPV5 and HPV8 were first identified in individuals afflicted with the hereditary syndrome epidermodysplasia verruciformis (EV) which is characterized by extensive and recalcitrant skin warts which can progress to non-melanoma skin cancer (NMSC) in sun exposed areas [368, 369]. NMSCs associated with  $\beta$  HPV also occurs in HIV+ or solid organ transplant patients with immune suppression [370-372], but in otherwise healthy individuals their etiologic role remains controversial [366].

Demonstration of the etiologic role of HPV16 and HPV18 in 50% and 20% respectively of cervical cancer cases globally has driven the development and licensure of prophylactic HPV vaccines. These vaccines, based on L1 virus-like particles (L1-VLP), effectively protect naïve patients from HPV infection and development of precursor lesions of anogenital cancer [148, 149, 153, 154]. However, L1-VLP vaccines afford type-restricted protection, driving the development of a 9-valent vaccine to broaden coverage to most of the common hrHPV [5] as well as candidate vaccines based upon the minor capsid protein L2, a conserved protective antigen. Unfortunately, given the challenges of global implementation of HPV vaccination, the prevalence of hrHPV infection remains high, especially among older unvaccinated patients in developed countries and of all ages in low resource countries [373, 374].

The L1-VLP vaccines provide no therapeutic benefit for those already infected [375], and there are no HPV-specific treatments available. Natural history, laboratory-based and histopathology studies suggest the ability of the immune response to control most HPV infections. Notably, half

of cervical hrHPV infections in immune competent women, even HPV16 and 18, become undetectable by DNA testing of cervical swabs within six months, consistent with immunologic control [376, 377]. However, it is also unclear whether clinical regressions of HPV disease and loss of the detection of viral DNA reflects the complete elimination of the virus or rather the restriction of the virus to small reservoirs in basal cells with potential for recrudescence upon immune senescence, HIV co-infection or active suppression. Indeed, re-activation of previously suppressed infections, rather than acquisition of new infections, has been proposed to drive a second peak in cervical cancer incidence in older peri-menopausal women [378, 379]. Likewise immune suppression caused by progressive HIV co-infection or deliberately in transplant patients is associated with dramatically elevated risk for HPV-associated cancer [370-372].

These observations suggest therapeutic vaccination has promise for control of established infections and HPV-associated disease, including possibly cancer [380, 381]. The development of such therapeutic vaccines requires a suitable model, but unfortunately HPVs do not replicate in animals. The recently discovered MusPV1 (MmuPV1) is an attractive model because it replicates in laboratory mice [382]. MusPV1 can be propagated in nude mice and is skin-tropic [267, 383, 384]. However, MusPV1 does not produce disease in common inbred laboratory strains because of potent immunologic control complicating its use for vaccine studies.

Here, we show the potential of MusPV1 challenge of outbred immunocompetent SKH-1 mice as a model for the diverse disease outcomes of HPV patient populations, highlighting the role of genetic background. Further we characterize T-cell function and key epitopes driving control of MusPV1 in inbred C57BL/6 and Balb/c mice. We show the ability of systemic DNA vaccination to elicit such responses, and their capacity to home to the site of infection. Finally, utilizing profound immunosuppression and highly sensitive molecular detection of viral nucleic acid, we



determine immunologic parameters for control of papilloma resulting in complete elimination of MusPV1 or persistent viral reservoirs in the epithelium.

## **Materials and Methods**

### **Mice & Ethics Statement**

6-8 weeks old female C57/BL6 and Balb/c mice were purchased from Jackson Laboratories and SKH-1 elite mice from Charles River, Inc. All animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and with the prior approval of the Animal Care and Use Committee of Johns Hopkins University (MO12M223).

### **Cell Culture.**

CT-26 cell line expressing Balb/c murine MHC class I and 293 cell lines stably expressing either one or both C57/BL6 murine MHC class I (293<sup>Kb</sup>, 293<sup>Db</sup>, 293<sup>KbDb</sup>) were maintained in RPMI medium supplemented with 10% fetal bovine serum, 100U penicillin and streptomycin, 1U non-essential amino acids, 1mM Sodium Pyruvate (Gibco, Life Technologies, Grand Island NY). 293TT cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum, 100U penicillin and streptomycin, 1U Non-essential amino acids, 1mM Sodium Pyruvate (Gibco, Life Technologies, Grand Island NY). Lentivirus containing MusPV *E6* and *GFP* was prepared and used to infect TC-1 cells. Fluorescent detection of GFP expression was used as a surrogate marker for MusPV *E6*.

### **MusPV1 genomic DNA and MusPV1 viral gene DNA construct**

Plasmid pAsylum containing the entire MusPV1 genome and pShell expression vector encoding codon optimized MusPV1 L1 and L2 capsid genes were a kind gift from Chris Buck (NCI). MusPV *E6* and MusPV *E7* DNA sequences were obtained from the PaVE: Papilloma virus

genome website and were subsequently codon optimized in a fusion gene termed hCRTmE6E7L2. Likewise an open reading frame encoding human calreticulin (CRT) and codon-optimized full length MusPV1 E6 and E7 and MusPV1 L2 aa11-200 synthesized by BioBasic and inserted between the *EcoRI* and *NotI* sites of expression vector pNGLV4a (kindly provided by the University of Michigan's National Gene Laboratory). Individual CRT-MusPV gene plasmids were made whereby the MusPV-E6/E7 genes were subsequently PCR amplified from the hCRTmE6E7L2 plasmid while the MusPV-E1/E2/E4 genes were PCR amplified from the MusPV genome. Primer sets for each full length gene is listed in the supplementary data (see Table 15). Following amplification, each was cloned between the *EcoRI* and *NotI* sites of pNGLV4a. All plasmid constructs were confirmed by DNA sequencing.

#### **Quantification of MusPV genomic DNA and E1<sup>^</sup>E4 transcript**

The pAsylum plasmid is inserted into a unique *XbaI* site within the MusPV1 genomic clone. The primer set used to detect MusPV DNA by PCR was designed to bridge this *XbaI* site and is as follows: Forward 5'-GGTCAAAGGGCAGCGTCTA-3', Reverse 5'-TGCTTCCCCTCTTCCGTTTT-3'. Analysis of MusPV E1<sup>^</sup>E4 mRNA transcript by reverse transcriptase quantitative PCR (RT-PCR) was performed as in [384].

#### **Production, establishment, propagation and collection of MusPV1**

Generation of infectious mouse papilloma virions were performed as described as mentioned in [13]. To grow more MusPV1 virion stocks, additional mice (typically n=5 nude mice to produce more stocks) were challenged using the protocol developed by Handisurya *et al* [384] with 200  $\mu$ L of stock extract per mouse, corresponding to a dose of  $>10^{12}$  viral genome equivalents (VGE).

### **Chromogenic in situ Hybridization**

Custom RNA in situ hybridization probes (Advanced Cell Diagnostics, Inc.) were prepared to detect full-length *E6/E7* mRNA sequence of MusPV1. RNAscope® assays were performed using the RNAscope 2.0 FFPE Brown Reagent kit according to the manufacturer's instructions. Briefly, formalin-fixed, paraffin-embedded tissues sections (FFPE) mouse tail sections were pretreated with heat and protease prior to hybridization with probe. To ensure RNA integrity and assay procedure, adjacent sections were also hybridized with a probe to the endogenous housekeeping gene *ubiquitin*. After washing, an HRP-based amplification system was then used to detect the target probes followed by color development with DAB.

### **Immunohistochemistry**

CD3 was stained by following PowerVision Poly-HRP IHC Detection system protocol (Leica Biosystem). Briefly, FFPE sections were deparaffinized in xylene, followed by dehydration in graded ethanol. Antigen retrieval was performed by steaming specimens at 100°C for 20 min in Target Retrieval Solution (Dako) and subsequently washed in Tris-buffered saline with Tween 20 (TBST, 0.05% Tween 20). Endogenous peroxidase was blocked, by treatment of slides with Dual Endogenous Enzyme-Blocking Reagent (Dako) for 5 min at room temperature. Sections were covered with rabbit monoclonal CD3 primary antibody (ThermoFisher, RM-9107, 1:300) diluted with Antibody Dilution Buffer (ChemMate) and then incubated at room temperature for 45 min. Slides were then washed with TBST, followed by incubation with PowerVision Poly-HRP Anti-Rabbit IgG for 30 min at room temperature. After three washes in TBST, sections were treated with DAB chromogen (3, 3'-diaminobenzidine tetrahydrochloride; Sigma) for 20 min in the dark. Sections were counterstained with Mayer's hematoxylin (Dako), dehydrated with ethanol and xylene, and mounted permanently.

## **Peptides**

A panel of 20mer peptides, each overlapping by 15 amino acids, were generated by Genscript, Inc, (New Jersey) at a purity of  $\geq 70\%$  for both MusPV E6 (25 peptides) and E7 (20 peptides). These 20mers were then pooled into libraries of 5 peptides covering for E6 amino acids 1-40, 25-65, 50-90, 75-115 and 100-140, and for E7 1-40, 25-65, 50-90, and 75-110. 9mer peptides (overlapping by one amino acid) for E6 amino acids 89-104 and E7 66-80 were synthesized to further define their CD8 T-cell epitopes.

## **DNA vaccination via electroporation**

C57/BL6 or Balb/c mice were injected intra-muscularly with at the hind leg thigh muscle with 15 $\mu$ g of DNA in 30 $\mu$ l PBS. Subsequently, a pair of electrode needles was inserted into the muscle area surrounding the DNA injection site. Electrical pulses were delivered using the BTX electroporation generator (ECM830, BTX Harvard Apparatus, Holliston, MA). 8 pulses of 106 V was delivered within a 20ms pulse of 200ms intervals.

## **Intracellular cytokine staining and flow cytometry analysis**

For all characterization studies, cell lines that overexpressed the murine MHC class 1 alleles first seeded at  $5 \times 10^5$  cells/well/ml in a 24 well plate before being transfected with the relevant DNA plasmids. After 48 h incubation, the cells were mixed with  $10^7$  splenocytes from either vaccinated mice or wart-bearing mice that were undergoing regression in the presence of Golgi plug for approximately 12 hours before the cells were collected for cell staining and flow cytometry analysis. For characterization of optimal specific epitopes of MusPVE6 and E7,  $10^7$  splenocytes from either CRT-MusPV-E6 or CRT-MusPV-E7 immunized mice or CRT-MusPV-E6E7L2 were incubated with the peptide libraries of MusPV1 E6 or E7, or later with specific 20mers or 9mers in the presence of Golgi plug for 12 h before the cells were collected for cell staining and flow cytometry analysis. To further characterize the MHC restriction background for MusPVE6 or E7

in the C57/BL6 background, 293<sup>Kb</sup> or 293<sup>Db</sup> were pulsed with peptide and co-cultured with 10<sup>7</sup> splenocytes from either CRT-MusPV-E6 or CRT-MusPV-E7 immunized mice in the presence of Golgi plug for approximately 12 hours before being collected for intra-cellular staining and flow cytometry. In all cases, the steps for intracellular cytokine staining and flow cytometry were performed as previously described in [385].

### ***In vivo* antibody depletion experiments**

*In vivo* antibody depletions were performed as described previously in [386]. Briefly, mice were treated I.P with 100µg (1µg/ul) depletion monoclonal antibodies for CD4 (clone GK 1.5), CD8 (clone 2.43) or CD3 (clone 145-2C11) (BioXcell). Depletions were initiated 1 week prior to MusPV1 virion challenge. Depletion was confirmed using flow cytometry. Cages were also changed every week to ensure no carry-over or contamination from residing virus when depletion studies were being maintained.

### **Establishment of murine MusPV E6-specific CD8 T-cell line.**

5 ~ 8 weeks old female C57BL/6 mice were vaccinated with 25µg of pcDNA3-CRT/MusPV E6 via intramuscular injection followed by electroporation. Splenocytes were prepared and stimulated with irradiated TC-1/MusPV E6 cells at the presence of murine IL-2 (20 IU/ml). The cells were re-stimulated once a week at the presence of murine IL-2. The specificity of the CD8 T-cells were determined by the stimulation with MusPV E6 peptide in the presence of GolgiPlug and followed by CD8 and IFN-γ intracellular staining.

## **Results**

### **Control of MusPV1 by cellular immunity**

Consistent with other studies [139, 337, 364], MusPV1 infection of immunocompromised mice resulted in florid papilloma and production of infectious MusPV1 (Figure 1D). In contrast, no

papillomas were observed in two commonly utilized immune-competent inbred mouse strains, Balb/c and C57/BL6, after challenge. However, upon challenge of out-bred female hairless SKH-1 mice, papilloma were observed in 3/20 mice (Figure 29A) despite their immune-competence and the induction of a MusPV1 L1 VLP-specific antibody response (data not shown). The papilloma on 2/3 of these mice subsequently regressed leaving a single mouse with persistent disease even after 6 months post infection (Figure 29B) with papillomas spreading to its muzzle (Figure 1B). This mouse was sacrificed and the papilloma were harvested for extraction of RNA and qRT-PCR testing for MusPV1 E1<sup>E4</sup> transcripts. MusPV1 E1<sup>E4</sup> transcripts were detected confirming the association with an active MusPV1 infection (Figure 29C).

In a repeat study with 20 male and 20 female SKH-1 mice, 9/40 mice developed papilloma (4 female, 5 male). The papilloma regressed on two mice of each gender within 3-5 weeks. However, even after 6 months, no regression was observed in the remaining 5; the papilloma continued to grow and spread along the tail and muzzle (data not shown). The SKH-1 mice with extensive papilloma burden from the second round experiment were subsequently bred together in order to query whether their offspring would have an increased likelihood to develop papilloma. However, none of the four litters exhibited clinically apparent papilloma and an antibody response toward MusPV1 L1 VLP was detected at 1 week and 10 weeks post weaning (data not shown).

Nude or SCID mice were also challenged concurrently with the SKH-1 mice to ensure that there were no technical issues with the mouse challenge, and all nude or SCID mice developed papilloma. Within 4 months of challenge, the papilloma on the tail of SKH-1 mice mostly remained localized to the challenge site on the tail. Although spreading of papilloma did eventually occur in a minority of SKH-1 mice, they never achieve the size of papilloma seen in nude or SCID mice, even after 4 months post infection (Figure 29B vs. 29D).

### **CD4 and CD8 T-cells individually control papilloma formation**

To assess which arms of the immune system contribute to control of MusPV1 infection, C57/BL6 mice deficient in CD4, CD40 ligand or Type I interferon or animals were challenged (Table 14). In addition, immunocompetent Balb/c were also individually depleted of CD4 T-cells, CD8 T-cells or neither, were also challenged with MusPV1. Surprisingly, no papilloma were observed in the six months after challenge in any of these depleted or knock-out mouse groups (Summarized in Table 14) despite the consistent induction of papilloma on contemporaneously challenged nude mice. This result is consistent with a recently published study by Harisundrya *et al* [383].

Based on our results, we hypothesized that both CD4 and CD8 T-cell populations individually could compensate for the other group and control for papilloma formation. To test this, we repeated the challenge study including Balb/c and C57/BL6 mice subjected to T-cell depletion with CD3 antibody (Clone 145-2C11). By 3-5 weeks following depletion and viral challenge, papilloma were observed on the tails of the CD3-depleted mice, irrespective of strain and similar to nude mice (Figure 36), whereas none were seen on consistently either the CD4 or CD8 antibody depleted mice.

To assess immune control of established MusPV1 papilloma, 10 Balb/c mice were CD3 T-cell depleted and challenged. Warts appeared 3-5 weeks post-infection. To assess if restoration of the T-cell population would lead to papilloma regression, (Figure 37A), CD3 T-cell depletion was maintained in five of these mice and halted in the remaining five. Upon cessation of depletion, the papilloma remained visible during the first 4 weeks, presumably due to the delay in reconstitution of the T-cell population and its activation. By weeks 5 and 6, the papilloma began to shrink and by week 10, the papilloma were no longer visible (Figure 37C). In contrast, the papilloma in the mice with ongoing CD3-depleted continued to grow and become florid (Figure 37B). This result

demonstrates the ability of T-cells to control even established disease and is once more consistent with a recently published study by Harisundrya *et al* [383]. To further assess the abilities of cell-mediated immunity,  $5 \times 10^6$  splenocytes from naïve Balb/c mice were adoptively transferred into papilloma-bearing SCID mice each (n=3). Papilloma regression occurred more slowly (approximately 3 months), possibly due to the low amount of immune cells transferred, delayed engraftment and subsequent activation and expansion. Immunohistochemistry staining with CD3 antibody revealed infiltration of the regressing papilloma with T-cells (Figure 2A), whereas none were observed in the papilloma of SCID mice that did not undergo any adoptive transfer (Figure 30B). These results suggest specific immune cells were able to traffic to the site of MusPV1 infection/disease and effect papilloma clearance

#### **Reactivation of asymptomatic infection by immune-suppression**

As HPV-associated disease is more recalcitrant and progressive in both solid organ-transplant (OTR) patients and HIV+ individuals with low CD4 T-cell counts [363], it was surprising that C57/BL6 or Balb/c mice depleted of either CD4 or CD8 T-cells failed to develop papilloma post MusPV1 challenge. We hypothesized that the absence of observable papilloma might not mean the absence of persistent infection. Indeed, Maglennon *et al* previously showed in immune competent rabbits using Rabbit Oral Papillomavirus (ROPV) that following papilloma regression, there was persistence of ROPV genome in the basal epithelial of the site of infection [387, 388]. To assess if the same phenomenon also occurred in the MusPV1 model, we modified our T-cell depletion experiment to test for the presence of MusPV1 genomic DNA on the 5 weeks post-challenged tails of control and CD3, CD4 or CD8 antibody depleted mice or immune-compromised mice (e.g nude) via skin swab followed by qPCR analysis (Figure 31A). The study was repeated three independent times using n=5 mice per group with consistent findings whereby MusPV genomic DNA was consistently detected upon swabbing CD4 or CD8 antibody depleted mice but were approximately  $10^4$ -fold lower than immunosuppressed mice that bore papillomas.



Conversely, control mice with wildtype immunity constantly exhibited  $>10^6$ -fold lower viral load than wart-bearing immunocompromised mice (Figure 31B). The viral loads seen in the CD4- and CD8- depleted groups suggest persistent, but clinically inapparent infection (Figure 31B). Importantly, a similar trend was observed for our CD4 knock-out mice in the C57/B6 background whereby it harbored the highest viral load compared to other immune-compromised C57/B6 background strains which also did not have any visible papilloma post challenge (data not shown). However, it was not clear whether the low level of virus detected by qPCR in swabs of the challenged wild type mice reflected a very low level of persistent infection or remnants of the challenge inoculum remaining in the cage environment despite ongoing changes of bedding.

Subclinical papillomavirus infection has been proposed to reside in the basal cell layer in other animal models [387, 388]. To confirm the presence of MusPV in the basal cells in this model, a highly sensitive chromogenic RNA *in situ* hybridization technique (ACD RNAscope) was utilized on the tail specimens of the challenged mice in place of qRT-PCR because of its potential to identify the cell types harboring clinically inapparent MusPV1 infection. Detection by a probe targeting E6/E7 transcripts was utilized because they are, unlike E1<sup>E4</sup>, considered to be expressed in all infected cells.

As expected, high levels of E6/E7 transcripts were present in the specimens from either nude or CD3-depleted wart-bearing mice mostly concentrated in the lower epithelial levels (Figure 31C). Interestingly, certain localized sections of tail specimens from CD4-depleted mice showed the presence of MusPV E6/E7 transcripts even in the absence of apparent papilloma (Figure 31D). However, evidence of MusPV1 infection was not seen in the basal layer in CD8 T-cell depleted mice despite presence of viral genomic DNA in the qPCR experiments and their histological results were similar to our findings in wildtype Balb/c or C57/BL6 tail specimens whereby no

trace of MusPV E6/E7 transcript was detected by RNAscope over the 10 tails suggesting that the virus was cleared by 10 weeks post challenge (Figure 31E).

To further characterize if the RNAscope of MusPV E6/E7 transcripts failed to detect transcriptionally-inactive MusPV1 virus in the mice without evident staining, we sought to reactivate viral reservoirs by switching the mice to full immunosuppression. Thus, following CD4, CD8 or no antibody depletion for 5 weeks post MusPV1 challenge, all mice were then switched to CD3 antibody depletion for a further 10 weeks to allow for re-activation of any persistent MusPV1 infection (summarized in Figure 32A). Cages were changed each week to limit fomite contamination or carry-over of virus. Following 10 weeks of CD3 depletion, 14/15 of the previously CD4 antibody depleted mice, but only 1/15 CD8-depleted mice grew localized warts (Figure 32C,D). These findings are in accordance with the persistent presence of MusPV E6/E7 transcripts and viral load in tail swabs (Figure 31B). Of note, none of the infected wildtype mice grew warts after CD3 depletion for 10 weeks suggesting complete clearance of infection in mice with intact immunity (Figure 32B) and is also consistent with the earlier viral load skin swab experiments (Figure 31B).

### **CD8 T-cell recognition of MusPV1 E6 and E7 in C57/BL6**

While CD8 T-cells contribute to papilloma regression [267, 383, 384], the relevant MusPV1 epitopes have not been characterized. To this end, DNA vaccines based on fusion of human calreticulin (CRT), an endoplasmic reticulum resident protein that potentiates antigen presentation via MHC I of linked epitopes, to full length MusPV1 E6 (CRT/mE6) or E7 (CRT/mE7) or E6, E7 and L2 (CRT/mE6E7L2). These viral antigens were selected because the obligatory expression of E6 and E7 in papillomavirus disease, immunotherapeutic activity in the HPV16 system using analogous constructs and the potential of L2 to elicit neutralizing antibodies [389-391].

Groups of 5 mice from both Balb/c and C57/BL6 were vaccinated weekly with either CRT/mE6, CRT/mE7 or CRT/mE6E7L2 for 3 weeks. A week after last vaccination, splenocytes were harvested and T-cell activation assays were performed (Figure 33A). For initial screening, we co-cultured these splenocytes respectively with either 293<sup>Dbkb</sup> cells or CT26 cells which over-express the respective murine MHC class 1 molecules for both genetic strains. These cells were transfected with CRT-alone, CRT/mE6 or CRT/mE7 to characterize if any CD8 T-cell response was elicited by these vaccines. A strong mE6 T-cell response was observed in splenocytes harvested from C57/BL6 mice vaccinated with CRT/mE6 or CRT/mE6E7L2, whereas there was no immune response detected against mE7 in either (data not shown). Surprisingly, no detectable cellular immune responses against either mE6 or mE7 were observed in the Balb/c background (data not shown).

As the results in Balb/c background could be due to insensitivity issues with the assay, we repeated our CD8 T-cell activation assays using 20mer over-lapping peptide libraries derived from MusPV1 E6 and E7 amino acid sequences. Once more a strong mE6-specific CD8 T-cell response was again detected in splenocytes of C57/BL6 mice vaccinated with CRT/mE6 (Figure 33B) or CRT/mE6E7L2 (Figure 33C), now a weaker mE7-specific CD8 T-cell response could be detected in splenocytes of mice vaccinated with CRT/mE7 (Figure 38A). Interestingly the mE7-specific CD8 T-cell response was still not detected in the splenocytes of CRT/mE6E7L2 vaccinated mice (Figure 33C), suggesting immune-dominance of mE6 over mE7 (Figure 33C). However, no mE6 or mE7 T-cell responses could be detected in the splenocytes of vaccinated Balb/c mice despite using these peptide libraries (data not shown). This suggests that cellular response in mediating papilloma regression in Balb/c mice is possibly directed towards other early proteins.

**The MHC class I immuno-dominant epitopes of MusPV E6 (aa90-99) and MusPV E7 is (aa69-76) are both H2<sup>Kb</sup> restricted for C57/BL6 mice**

Given our results, in our initial T-cell studies, we decided to focus on C57/BL6 mice. The use of pools of 20mer overlapping mE6 and mE7 peptides suggested their dominant epitopes lie within aa75-115 of mE6 (Figure 33B, 33C) and within aa50-90 of mE7 (Figure 38A). To determine the specific epitope sequence, we designed several 9mer epitope peptide candidates that overlap by one amino acid for mE6 within region aa89-104 and mE7 within aa66-80. T-cell activation assays were then performed using splenocytes from either CRT/mE6 or CRT/mE7 vaccinated mice and stimulation with their respective 9mer candidates. The immunodominant epitope of MusPV1 E6 was localized at a.a. 90-99 (KNIVFVTVR) (Figure 33D) and in MusPV1 E7 at a.a. 69-77 (VLRFIIVTG) (Figure 38B). Following elucidation of these epitopes for C57/BL6, we examined which H-2<sup>b</sup> MHC class I molecule presents these epitopes by co-culture of splenocytes from either CRT/mE6 or CRT/mE7 vaccinated mice with either 293<sup>Kb</sup> or 293<sup>Db</sup> cells transfected with their respective viral antigen or CRT alone as a control. Results showed that the MHC class I epitope of mE6 and mE7 are both H-2K<sup>b</sup> restricted in the C57/BL6 background (Figure 33E and Figure 38C).

**Spontaneous mE6-specific CD8 T-cell response correlates with MusPV1 papilloma clearance in C57/BL6 mice**

It is possible that epitopes in other MusPV1 proteins are recognized by CD8 T-cells during natural regression. Further, given the negative results for Balb/c in our peptide library studies with mE6 and mE7, this seemed even more possible. To assess this possibility, CD3-depletion of a group of 5 C57/BL6 and 5 Balb/c mice bearing florid MusPV1 papilloma was stopped. Once the papilloma were completely regressed, splenocytes were harvested from both groups and respectively pooled. Splenocytes were incubated with either 293<sup>DbKb</sup> or CT26 cells, which over-

express the C57/BL6 and Balb/c MHC class I molecules respectively, which were transfected with expression vectors for either hCRT-alone, or hCRT-linked to either MusPV1 E1, E2, E4, E6, E7, L1 or L2 respectively. To control and account for potential technical sensitivity issues with the transfection assay, the C57/BL6 splenocytes were also stimulated directly using the MusPV1 E6 a.a. 90-99 (KNIVFVTVR) and E7 a.a. 69-77 (VLRFIIVTG) peptide epitopes. A specific mE6 specific CD8 T-cell response could only be clearly detected in the splenocytes of C57/BL6 mice that had spontaneously cleared their papilloma and interestingly, there was also a weak L1 T-cell response (Figure 39). However, no MusPV1 E7-specific CD8 T-cell response was detected in the C57/BL6 mice, even when the splenocytes were directly stimulated with the mE7 peptide. Likewise, no response against the other MusPV1 full length viral proteins was detected. Unexpectedly, no CD8 T-cell responses were detected for Balb/c again (data not shown), suggesting a potential lack of sensitivity in the assay due to weak antigen expression or presentation. Nonetheless, our results suggest that E6 is the dominant antigen in C57/BL6 mice that produces a specific CD8 T-cell response against MusPV1.

### **Adoptive transfer of E6-specific T-cells prevents the development of papilloma in MusPV1-infected immunodeficient mice**

The CRT-mE6 or CRT-mE6E7L2 vaccine elicited an E6 a.a. 90-99 specific CD8 cytotoxic effector T-cell response similar to that present in C57/BL6 mice after spontaneous clearance of MusPV1 papilloma. To examine whether this response is sufficient to clear an established infection to prevent subsequent papilloma formation, we expanded for adoptive transfer experiments a MusPV E6 a.a. 90-99 specific CD8 cytotoxic T-cell line *in vitro* (Figure 40) from the splenocytes of C57/BL6 mice previously vaccinated with CRT-mE6 DNA three times by *in vivo* electroporation. Briefly, RAG1-KO mice (n=10) were first challenged with MusPV1 using a dose sufficient to produce papilloma within 3-5 weeks. A week later following challenge, the mice were divided whereby 5 of the mice were administered the MusPV E6-specific CD8 T-cell

line *i.v.* ( $5 \times 10^6$  T-cells), while the 5 controls were administered  $5 \times 10^6$  OT-1 T-cells, an ovalbumin (OVA)-specific CD8 T-cell line (Figure 34A). By week 4, the tails of the mice that received the MusPV E6 specific CD8 T-cells had healed and lacked papilloma (Figure 34B). Conversely, the tail of each mouse treated with OT-1 cells had developed small papilloma (Figure 34C). Further analysis of these tails by RNAscope in situ hybridization with the MusPV1 E6/E7 probe revealed strong staining in the papilloma of the mice treated with OT-1 cells after MusPV1 challenge (Figure 34C), whereas the tails of mice treated with adoptive transfer of the MusPV E6-specific CD8 T-cell line were devoid of signal, suggestive of viral clearance or profound suppression of early transcription (Figure 34B). Thus adoptive transfer of the MusPV1 E6-specific CD8 T-cell line generated by DNA vaccination and *in vitro* expansion can prevent the formation of papilloma in MusPV1 infected immunodeficient mice.

#### **Clearance of established papilloma and suppression of MusPV1 infection in immunodeficient mice by adoptive transfer of E6-specific CD8 T-cell line**

To examine impact on established disease, 8 RAG1-KO mice of the C57/BL6 background were challenged with MusPV1 and infection was maintained for 5 weeks until all had developed visible papilloma. Subsequently, four mice were administered  $5 \times 10^6$  CD8 T-cells of MusPV E6-specific line *i.v.* while four received  $5 \times 10^6$  OT-1 cells (summarized in Figure 35D). The papilloma on those mice treated with OT-1 cells continued to grow and become increasingly florid (Figure 35, bottom panel), whereas the papilloma on mice treated with the MusPV1 E6-specific CD8 T-cell line stabilized for 3 weeks, shrank significantly by weeks 6-8 (Figure 35, top panel) and were not visible by 10 weeks post treatment. To assess if viral clearance was achieved, the mice were subsequently sacrificed and their tails were analyzed via RNAscope. Results once more revealed strong staining in the papilloma of the mice treated with OT-1 cells (Figure 35B). However, in some sections of the tails of the mice that received the MusPV E6 specific CD8 T-cell line, traces of MusPV1 *E6/E7* transcript were detected (Figure 35C). However the level was

extremely low compared to the control papilloma-bearing mice. Thus the results suggest MusPV E6-specific CD8 T-cells can, upon adoptive transfer i.v., traffic to papilloma, mediate clinical remission and strongly suppress viral transcription by 10 weeks and are suggestive that T-cells developed by systemic vaccination potentially are able to traffic to the papilloma and mediate regression

### **Expansion of E6-specific CD8 T-cells in MusPV1 infected mice is sufficient to mediate clearance of persistent infection or disease**

Following completion of the adoptive transfer studies from both the papilloma-protection (Figure 34) and papilloma treatment (Figure 35) experiments, the mice from each group were bled and splenocytes harvested. Splenocytes from each group were pooled before stimulation in triplicate with either MusPV1 E6 peptide or OT-1 peptide. Intracellular cytokine staining for IFN- $\gamma$  revealed a CD8 T-cell population specific for MusPV1 E6 in the spleens of mice that were protected from (Figure 34E) or cleared established papilloma (Figure 35E). Conversely, no OVA peptide-specific CD8 T-cell response was detected in the spleens of mice that had undergone adoptive transfer with OT-1 specific CD8 T-cells, suggesting that in the absence of cognate antigen they die. Conversely, the presence of papilloma, or even subclinical infection, provided sufficient antigen to support ongoing proliferation of adoptively transferred MusPV1 E6-specific CD8 T-cells. Serum of mice from each group were tested negative for MusPV1 anti-L1 antibodies (data not shown) suggesting that the clearance of virus or disease was purely T-cell mediated.

### **Discussion**

HPV-associated diseases are more recalcitrant and progressive in patients with an immune system compromised by hereditary genetics (EDV or WHIM), drugs to prevent organ transplant rejection or co-infection with HIV [366, 369]. In contrast, most immune-competent individuals clear HPV

infection, often without apparent disease, whereas others develop papilloma that are either slowly cleared, or in some persist and/or progress [392]. The availability of genetic knockout mice and a plethora of immunologic reagents for the laboratory mouse provide an important opportunity to model and dissect the mechanisms mediating control and/or clearance of MusPV1 infection, and presumably papillomaviruses in general.

Based upon our genetic knockout and antibody depletion studies in C57/BL6 and Balb/c mice, it is clear that CD4 and CD8 T-cell-dependent immunity each contribute to control MusPV1 (Table 14) and together can eliminate infection. Indeed depletion of both subsets is required for the emergence of papilloma after challenge (Figure 36), and when either CD4 or CD8 T-cell-dependent immunity is depleted, small reservoirs of virus remain in the challenged epithelia (Figure 31). To assess if these small reservoirs could be reactivated and produce disease, we used CD3 antibody to provide immune suppression following 5 weeks of individual T-cell population depletion with CD4 or CD8-specific antibody after MusPV1 challenge (Figure 32A). Upon CD3 antibody administration, papilloma grew in 14/15 of those mice first depleted with CD4 antibody. In contrast, none of the wildtype mice (n=0/15) and only n=1/15 mice in the CD8 depleted group developed papilloma following full immune suppression (Figure 32). These results suggest that when the cellular immune response is further compromised, clinically apparent disease emerges in the mice whose CD8 and especially CD4 T-cell-dependent immunity was compromised prior to MusPV1 challenge. However, it is important to recognize that the CD8 T-cell depleted mice, unlike the CD4 T-cell depleted mice, were able to mount a neutralizing antibody response, that this may restrain the re-emergence of disease after CD3 T-cell-depletion (Figure 41).

In rabbits, Maglennon et al observed longterm persistence of ROPV DNA after clearance of papilloma and that immunosuppression with cyclosporine and dexamethasone could reactivate persistent asymptomatic papillomavirus infection to elicit papilloma [387, 388]. This contrasts the



complete clearance of MusPV1 DNA in C57/BL6 mouse and failure to recover papilloma after CD3 antibody depletion. However, the persistence of MusPV1 reservoirs in CD4 T-cell depleted mice after challenge, and papilloma growth upon further immunosuppression, is reminiscent of recalcitrant HPV infection in HIV+ individuals, including increased severity of disease and higher rates of HPV-associated cancer are reported as CD4 T-cell counts drop and AIDS progresses [370, 393]. Importantly, this highlights the contribution of CD4 T-cells in viral control, but the mechanism is less clear.

The CD4 T-cells may provide help for the induction of MusPV1-specific CD8 T-cells and/or the induction of L1-specific neutralizing antibodies, and possibly more direct effects including the release of anti-viral cytokines and cell killing. Mice with partial T-cell population which unable to produce antibody (e.g CD4 depleted mice) were still effectively control MusPV1 infection (Table 13 and Figure 41). However, while L1-specific neutralizing antibodies are not expected to act directly on infected cells, their presence can block the spread and reduce the load of virus within the host, as it re-emerges from viral reservoirs [394]. Indeed, in a recent study using L1-VLP vaccination in the MnPV model, the presence of L1-antibodies elicited by VLP vaccination prevents skin tumor development and progression after immunosuppression with cyclosporine of animals with established but controlled infection [394].

Interestingly, an immune response elicited in CD8 T-cell depleted mice was also able to clear MusPV1 from almost all mice. This observation raises the possibility of direct killing of the infected cells by a CD4 T-cell-dependent response, or that the antibody-based CD8 T-cell depletion was not sufficiently profound (although depletion was >95%) such that a small fraction remained to mediate clearance. However, CD8 knockout mice have been reported to also control MusPV1 infection [383].

Since the licensed HPV VLP vaccines lack demonstrated therapeutic activity, there remains a demand for medical treatments of persistent HPV infection and established disease. Preclinical studies in C57BL6 mice demonstrate that HPV16 E6- and E7 specific CD8 T-cell responses and anti-tumor immunity elicited by DNA vaccination are significantly enhanced when the antigen is linked to CRT via improved antigen presentation through the MHC class I pathway [390, 391, 395] and this occurs even in the absence of CD4 T-cell help. An ongoing trial is examining the safety and immunogenicity of the pNGLV4a-CRT/E7 DNA construct in women with high grade CIN (NCT00988559).

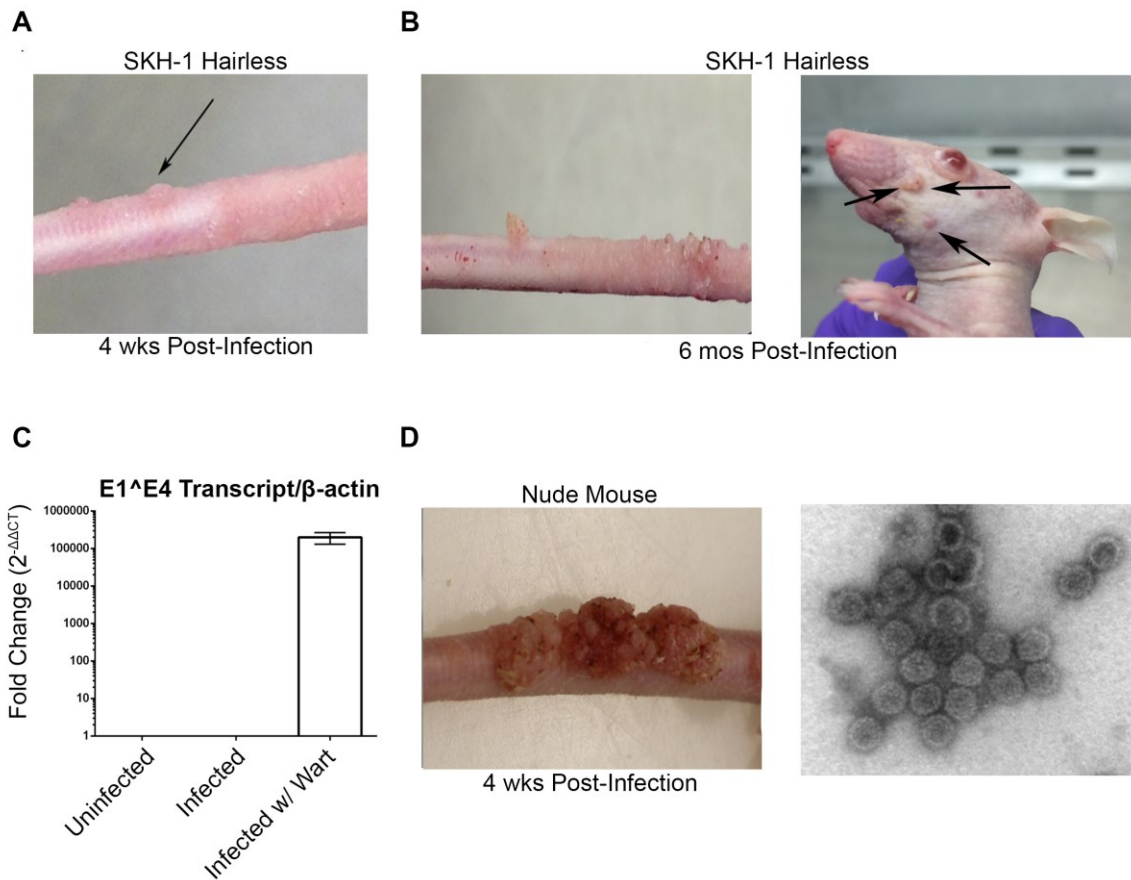
Using the same vaccine strategy for MusPV1 antigens, we show that DNA encoding CRT linked to either wildtype MusPV1 E6 (mE6) and MusPV1 E7 (mE7) induced specific CD8 T-cell responses in C57/BL6 mice (Figure 33). The MHC class I CTL immunodominant epitope for mE6 was at aa90-99 while a weaker mE7 epitope was mapped to aa69-77; both were H2-K<sup>b</sup> restricted (Figure 33 and Figure 38). Vaccination utilizing DNA encoding CRT linked to mE6, mE7 and MusPV1 L2 11-200 also was able to induce a potent mE6 aa90-99 -specific CD8 T-cell response in C57/BL6. However, no mE7 response was detected (Figure 33C) suggesting that the E6 response is dominant. In contrast, for the HPV16 version of this DNA vaccine consisting of HPV E6, E7 and L2, the dominant response is to E7 in C57/BL6 mice [391]. Further, IFN $\gamma$ /CD8 activation studies on the splenocytes taken from unvaccinated C57/BL6 mice that had naturally regressed papilloma detected a T-cell response only to mE6 (Figure 39). Taken together, these results suggest that in the C57/BL6 background, mE6 is the immune-dominant antigen and a relevant correlate of antiviral immunity. Interestingly, evidence points to HPV16 E6 being the dominant antigen in humans [396]. Neither CD4 nor CD8 T-cell responses to mE6 or mE7 in Balb/c were detected also after DNA vaccination despite several attempts. Given that papilloma regression was observed in this strain and clearance is potentially T-cell mediated (Figure 30), the failure to detect a mE6 or mE7 T-cell immune response in Balb/c is either due to technical issues

in peptide mapping design or the therapeutic response is directed to other viral proteins. Indeed several studies in animals and patients have suggested that E1 and E2 are potential rejection antigens [397]. Taken together, our results suggest that it may be beneficial to vaccinate against more than a single viral antigen to elicit therapeutic responses in a greater proportion of patients, but responses to a single epitope can dominate.

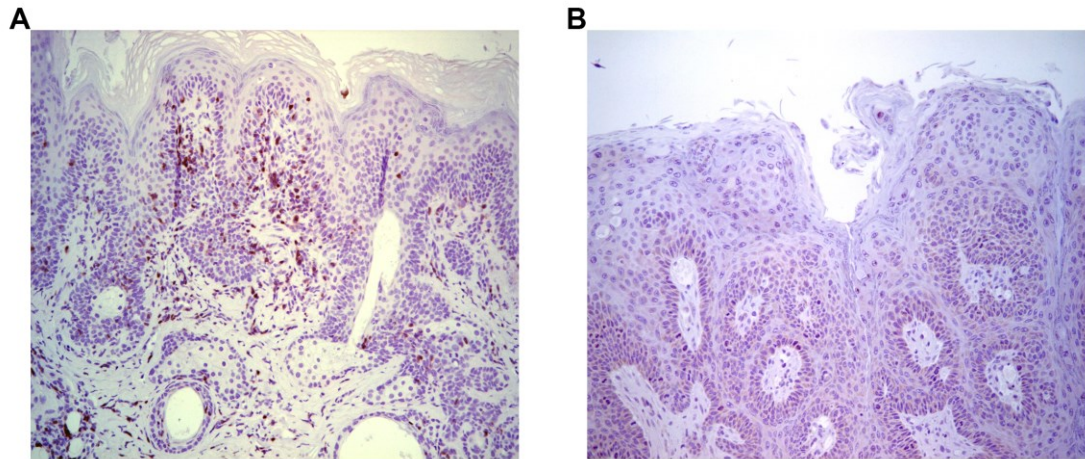
To assess if our MusPV1 DNA vaccine could produce an immune response that could clear persistent infection and disease, a CD8 T-cell line was developed from splenocytes of mice vaccinated systemically (by *i.m.* injection and electroporation) with a DNA vaccine expressing MusPV1 E6 fused to CRT by repeated *in vitro* stimulation with E6 a.a. 90-99 peptide (Figure 40). Importantly, we demonstrated protection from papillomatosis and clearance of MusPV1 infection when immunodeficient (RAG1- KO) mice received  $5 \times 10^6$  MusPV1 E6-specific CD8 T-cells by adoptive transfer one week after viral challenge (Figure 34). A similar experiment in mice with established papilloma demonstrated that these CD8 effector cells were potentially able to find, infiltrate, proliferate and eliminate disease within 8-10 weeks (Figure 35). However, low levels of virus remained detectable in certain areas of the tail sections at 10 weeks (Figure 35C) although it is possible with further time, these viral reservoirs would have been cleared subsequently. Nonetheless, these observations suggests that CD8 T-cell responses elicited by systemic vaccination can expand (Figure 34D, 35E) traffic to the site of infection, and that mucosal vaccination for local application of adjuvant is not required for effective homing, although they might be beneficial. More significantly, our therapeutic investigations using MusPV1 show that a substantial length of time is required to clear established papilloma and that even following lesion regression, viral reservoirs may remain (Figure 35A-C). In contrast, the therapeutic response was more effective against persistent infection in the absence of established disease (Figure 34).

Given the increasing use of HPV testing for screening, our results suggest the potential for therapeutic vaccination of persistent infection to prevent the onset of high grade neoplasia.

Our studies were consistent with others showing common inbred strains of mice fully control MusPV1 infection [267, 383]. However, for the first time, we show that challenge of the outbred SKH-1 mouse strain leads to the diverse outcomes seen in HPV-infected patients (Figure 29). A subset of the outbred SKH-1 mice were, and by implication patients too, are genetically susceptible and will develop persistent infections, whereas the remainder will clear their infections in time. An understanding of the genetic factors in patients driving the different outcomes might be applied to identify those that will clear the infection without intervention, and those needing interventions. The challenge of SKH-1 mice with MusPV1 represents a promising model in which to evaluate therapeutic vaccination. While challenge of inbred mice with syngeneic tumor models e.g. TC-1 provides critical mechanistic information, studies in outbred SKH-1 mice with MusPV1 that exhibit a range of clinical outcomes may be more predictive of clinical studies of vaccines and other immunologic interventions. Furthermore, the response to MusPV1 E6 is dominant, as suggested for HPV16 E6 in patients. Taken together, the SKH1 mouse studies clearly demonstrate the importance of genetic background, possibly reflecting histocompatibility and/or susceptibility to skin carcinogenesis, in the outcomes of MusPV1 challenge.

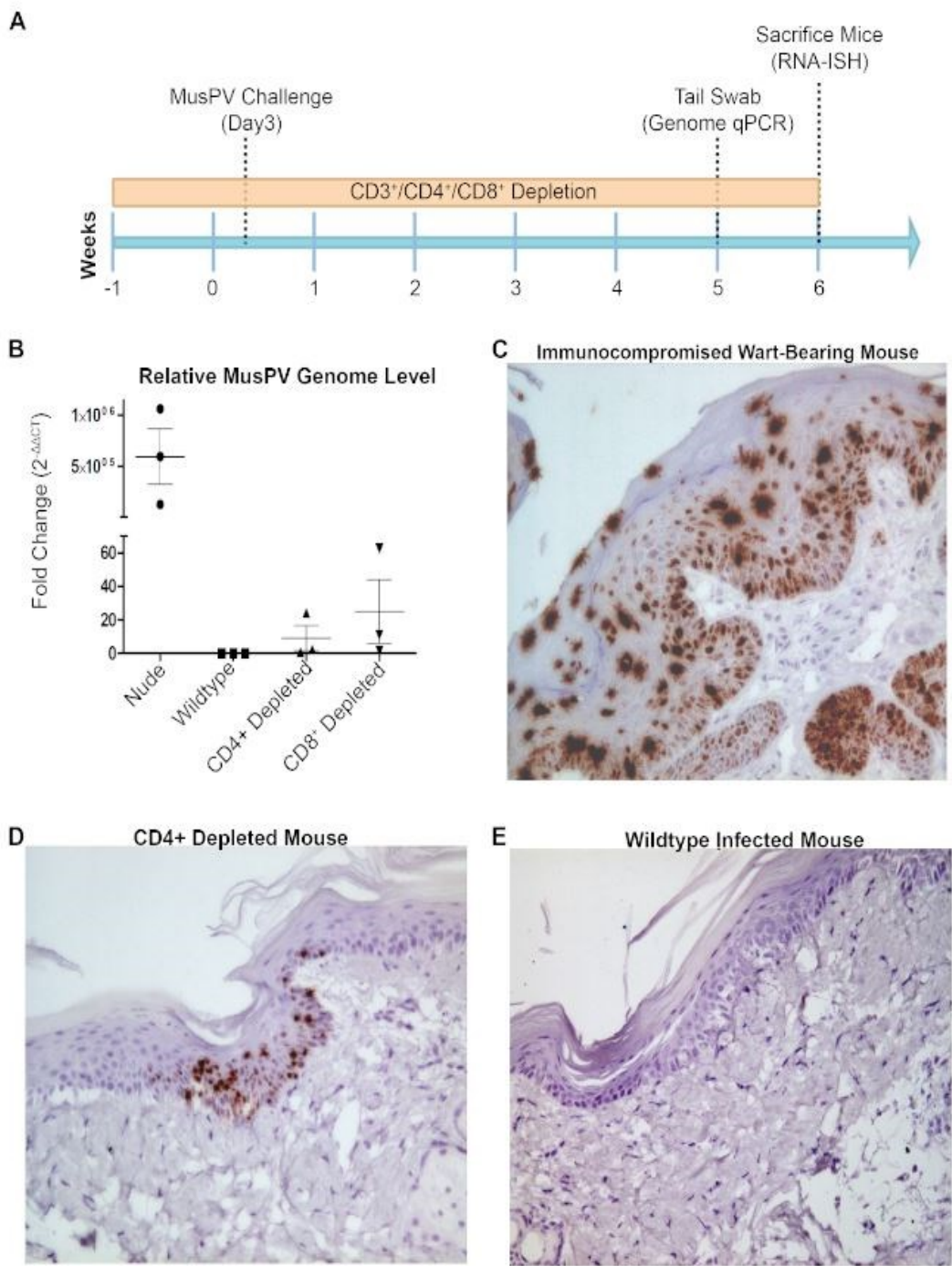


**Figure 29. MusPV1 infection and disease in outbred SKH-1 mice and immunocompromised controls.** Papilloma formation 4 weeks-post infection on the tail of a representative outbred SKH-1 hairless mouse (A). The papilloma persisted on the tail over 6 months and spread along the tail as well as to the muzzle (B). To test for active infection, papillomas were harvested and assessed for MusPV1 E1<sup>E4</sup> transcripts (C). Papillomas on SKH-1 mice were never as fluid compared to those on Nude mice (D). MusPV1 virions were harvested from the papillomas of nude mice and visualized using negative stain transmission electron microscopy (D).



**Figure 30. Infiltration of T-cells into papilloma site leads to MusPV1 papilloma regression**

Immunohistochemistry using CD3-specific antibody of papilloma in SCID mice that had received Balb/c splenocytes by 5 weeks post adoptive transfer and initiated papilloma regression (A) versus control SCID with progressive papilloma (B).

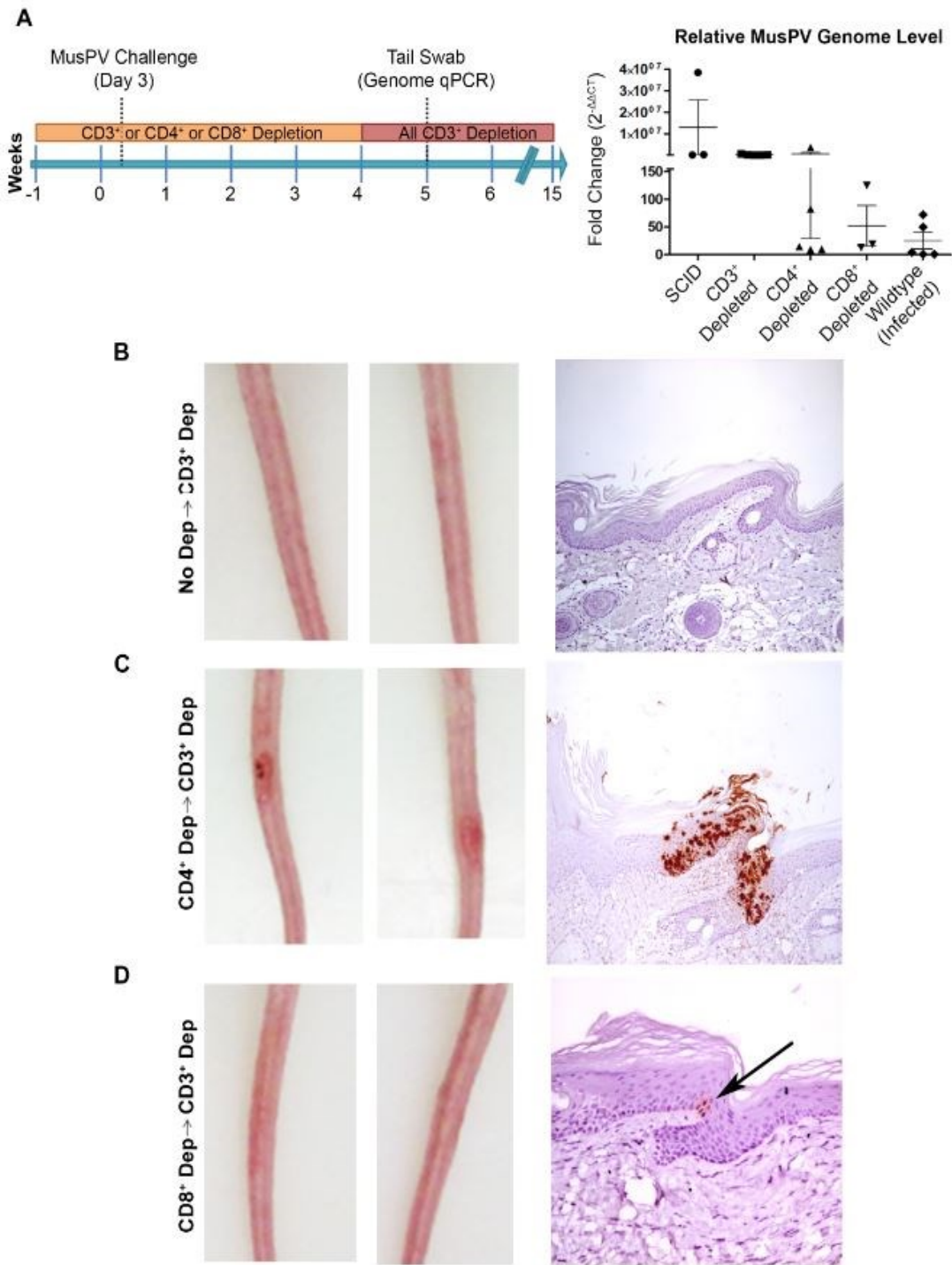


**Figure 31. Detection of persistent infection of MusPV1 despite absence of papilloma.**

Schedules of T-cell depletion using anti-CD4/CD8 monoclonal antibody and the timing of MusPV1 virus measurements in Balb/c mice with either anti-CD3 depleted or nude mice as control (A). Representative qPCR data from 3 independent experiments of MusPV1 viral

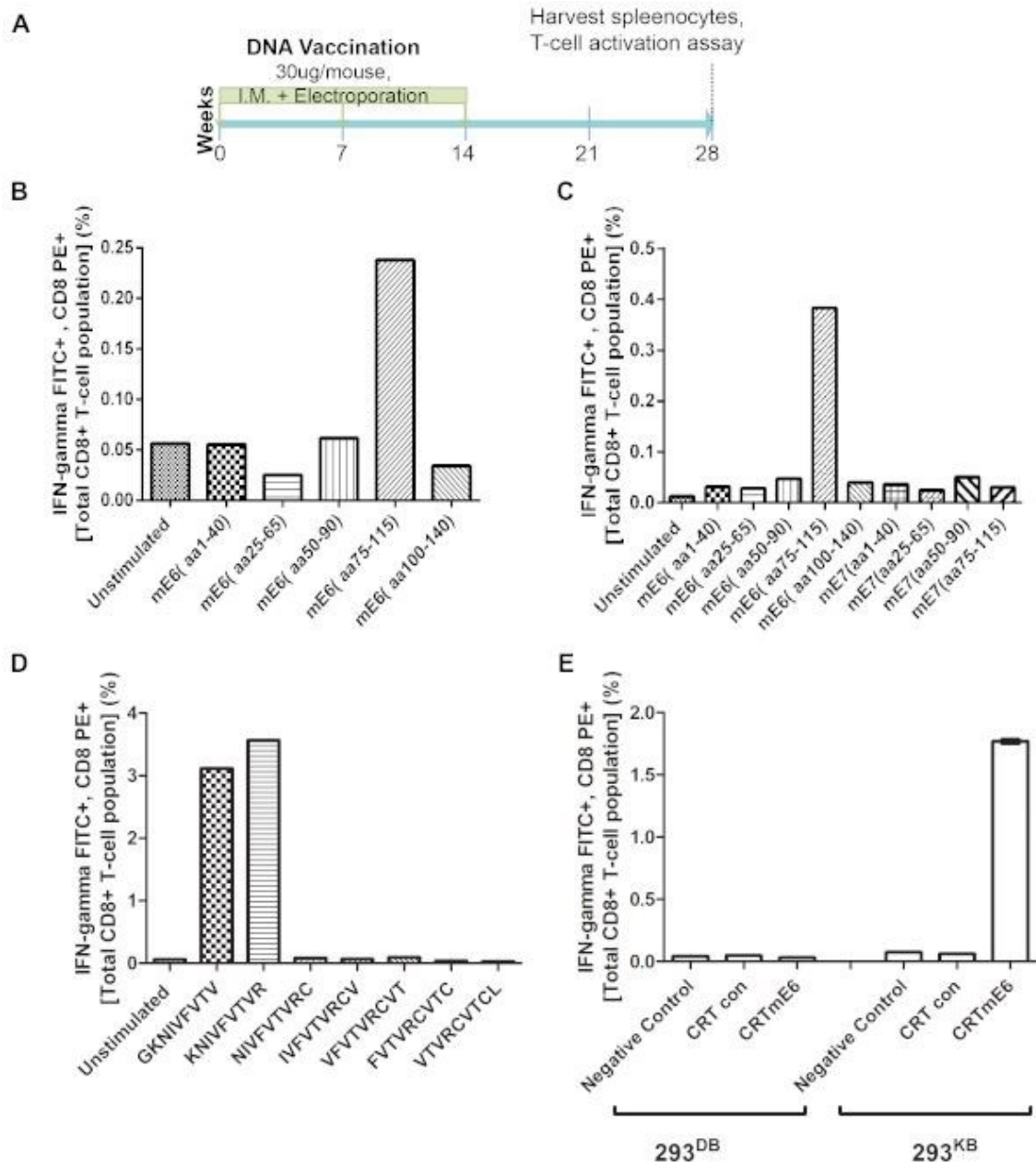
genomic equivalents with MusPV1-challenged wild type mice set as reference point 1.0 (B). RNAscope results showing production of MusPV E6/E7 transcripts in nude wart bearing mice (C) and to a lesser extent in CD4-depleted mice (D) but no transcripts observed in wildtype infected mice (E).





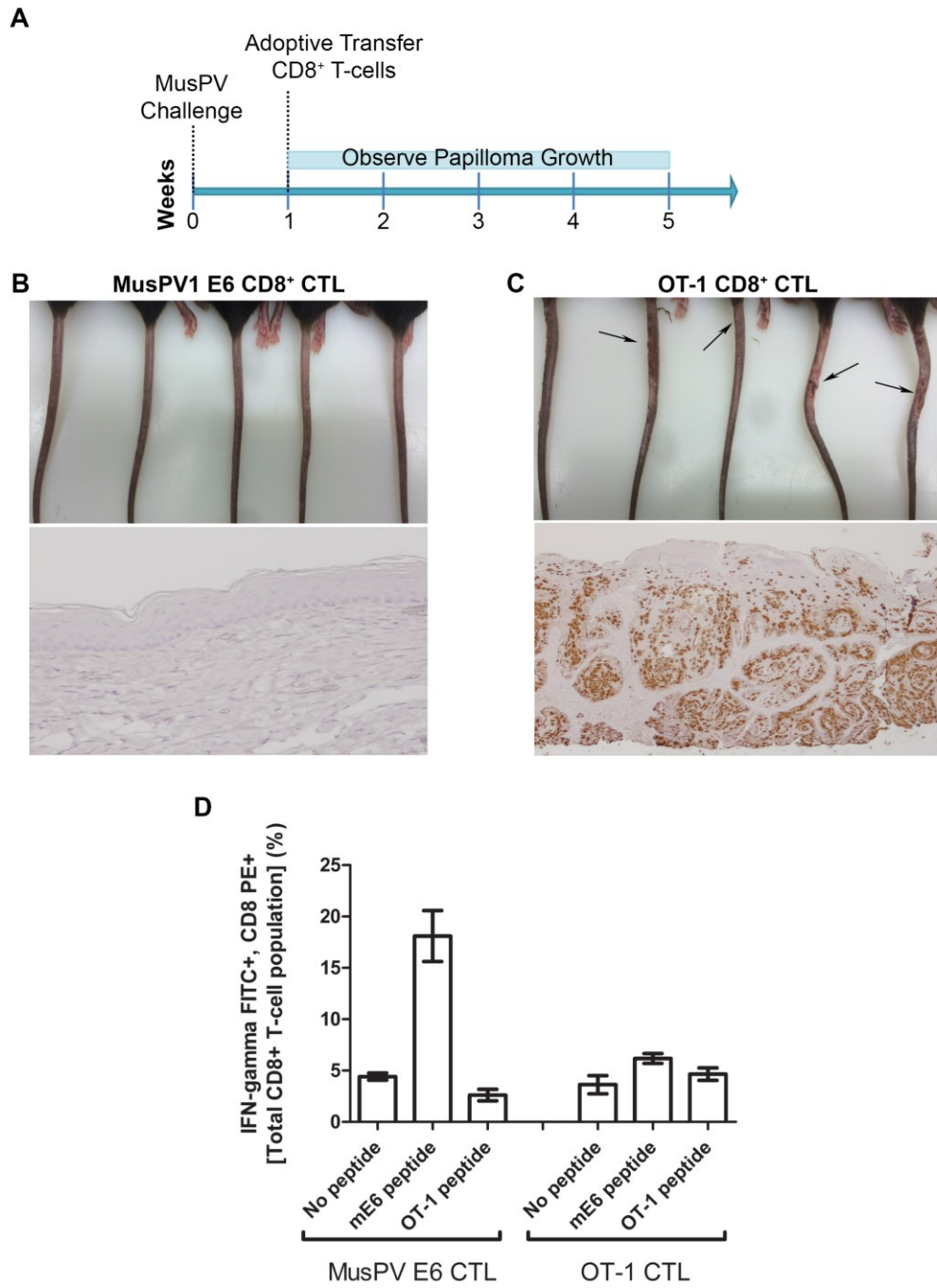
**Figure 32. Re-activation of MusPV1 infection.** Schema of experiment (left panel) in Balb/c mice, including schedule of T-cell depletion using monoclonal antibodies to CD3, CD4 or CD8, MusPV1 challenge to the tail, qPCR assessment of MusPV1 viral genome levels (right panel),

prior to CD3 antibody depletion of T cells (A). Representative images of mice tails and their histologic appearance after the final 10 weeks of depletion with CD3 antibody following without antibody depletion (B) CD4 antibody depletion (C) or CD8 antibody depletion (D) for 5 weeks initially. These respective mice were subsequently sacrificed and their tails were harvested and processed for in situ hybridization for MusPV1 E6/E7 using RNAscope and hematoxylin staining to obtain the histology images.



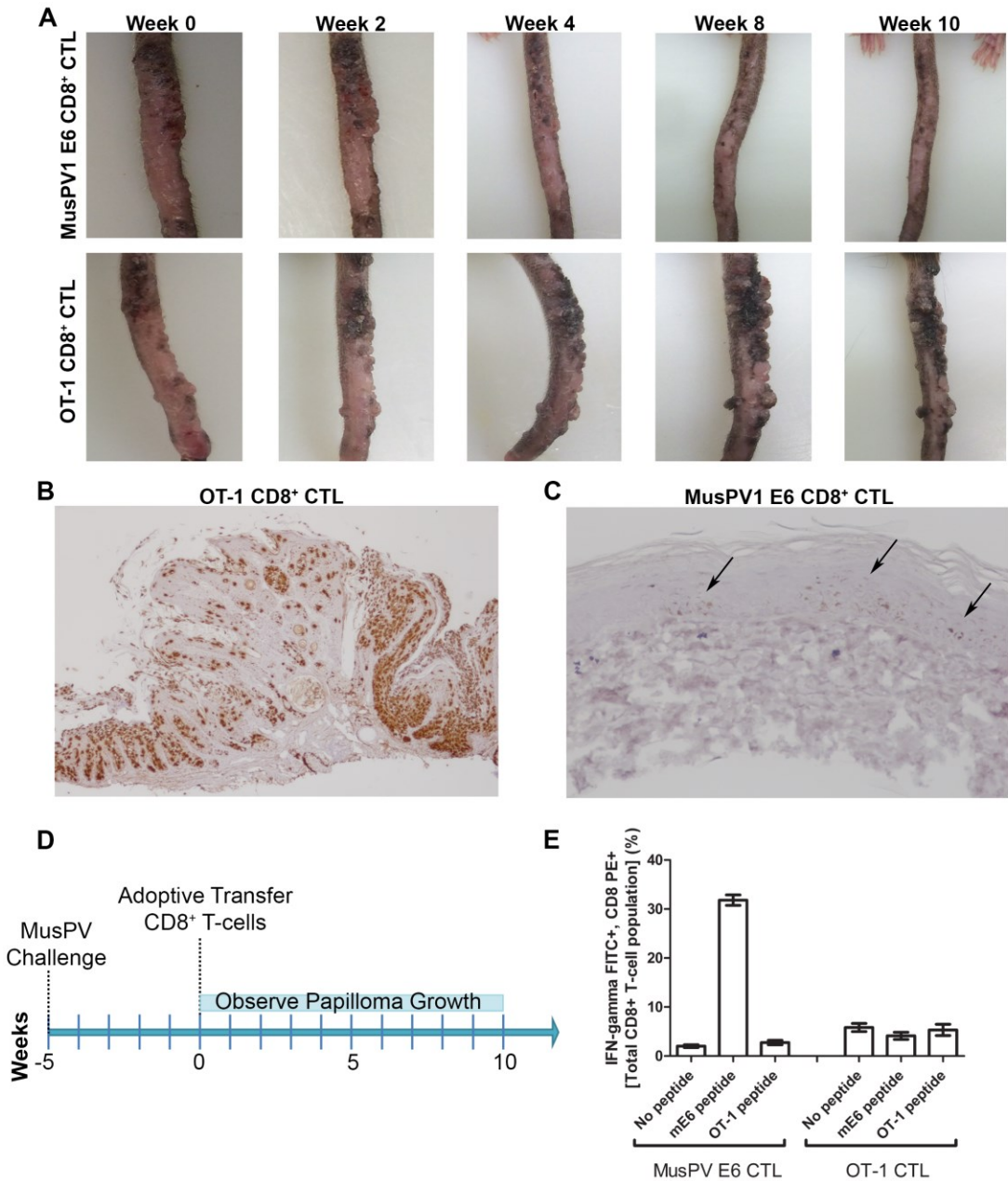
**Figure 33. Intracellular cytokine staining with flow cytometry analysis reveals immunodominant CD8+ T-cell epitope and MHC class 1 restriction of MusPVE6.** Schematic of immunization schedule of C57/BL6 mice with different MusPV1 CRT-linked DNA vaccine constructs and subsequent harvest of splenocytes (A). Bar graph of flow cytometry data after intracellular cytokine staining of splenocytes for interferon- $\gamma$  and CD8 after harvest from CRTmE6-vaccinated mice and stimulation with mE6 peptide library pools (B). Bar graph of flow

cytometry data after intracellular cytokine staining of splenocytes for interferon- $\gamma$  and CD8 after harvest from CRTmE6E7L2-vaccinated mice and stimulation with both mE6 and mE7 peptide library pools (C). Bar graph of flow cytometry data after intracellular cytokine staining of splenocytes for interferon- $\gamma$  and CD8 after harvest from CRTmE6 and stimulated with candidate 9mer peptides to map the MHC class I epitopes of MusPV1 mE6 (D). Bar graph showing flow cytometry data showing percentages of interferon- $\gamma$  expressing mE7 specific CD8<sup>+</sup> T cells co-incubated with 293 cells expressing either the Murine MHC class 1 molecule H-2K<sup>b</sup> (293- K<sup>b</sup> or 293- K<sup>d</sup>) that were transfected with either CRT-MusPV E6 or CRT-alone DNA plasmid vector to determine the MHC class 1 restriction. The results show that mE6 is K<sup>b</sup>-restricted (E). All data was repeated and representative images provided.



**Figure 34. Adoptive transfer of a MusPVE6-specific CD8<sup>+</sup> cytotoxic T-cell line one week after MusPV1 challenge prevents papilloma formation in immunodeficient mice. Schematic**

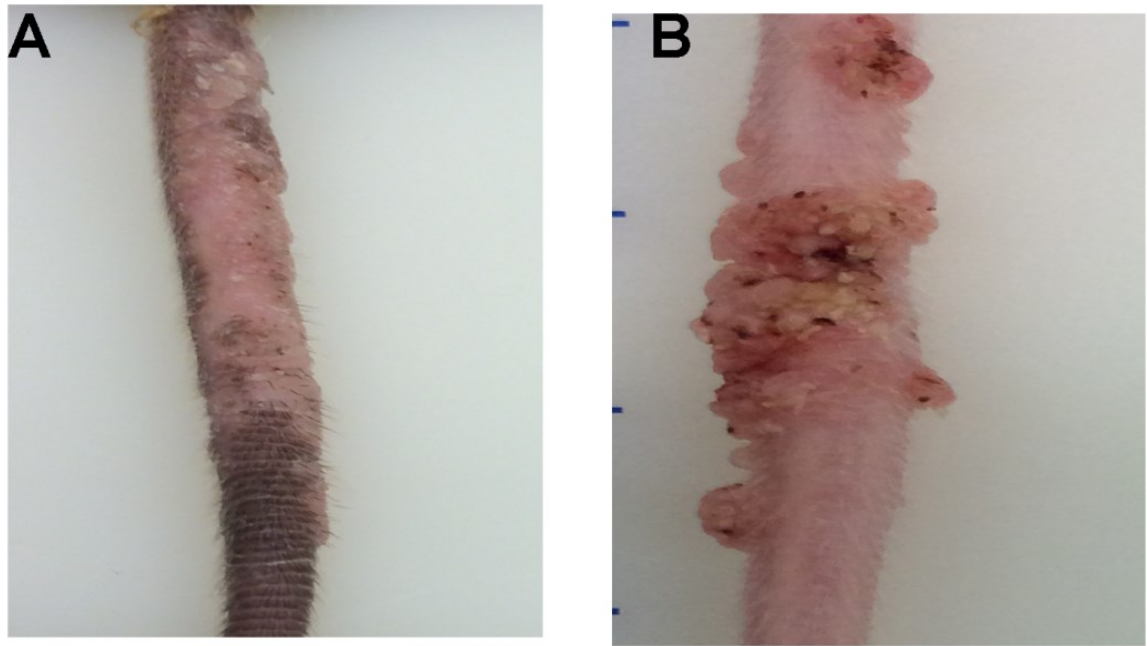
of study in RAG1 knock-out mice that received adoptive transfer of either  $5 \times 10^6$  CD8+ MusPVE6-specific T cell line, or  $5 \times 10^6$  OT-1 cells as a control via retro-orbital route, one week after MusPV1 challenge (A). Photographs and tail sections of MusPV1 E6/E7 in situ hybridization by RNAscope of RAG knock-out mice (n=5 per group) taken 5 weeks post-adoptive transfer with either the MusPV E6-specific CD8+ T cell line (B) or the OVA-specific OT-1 CD8+ T cell line (C). Presence and expansion of MusPV E6 CD8+ T-cells in the spleens of RAG knock-out mice who were protected from papillomatosis was apparent 5 weeks post adoptive transfer, but not OT-1 cells (D).



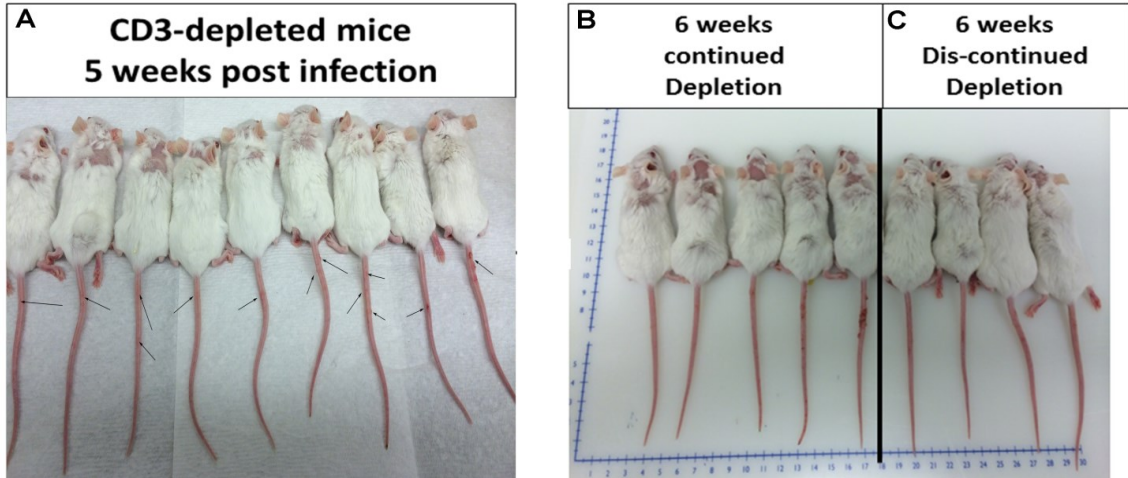
**Figure 35. Control of established papilloma by adoptive transfer of MusPVE6-specific CD8<sup>+</sup> T cell line.** RAG knockout mice, n=4 per group, were challenged with MusPV and papillomas were allowed to grow. After 5 weeks, the mice received by adoptive transfer either MusPV E6-specific CD8<sup>+</sup> T cells or OT-1 T cells. Mice were photographed every week thereafter for 10 weeks until the tails were harvested, sectioned and processed for MusPV1 E6/E7 in situ hybridization by RNAscope and hematoxylin staining. Photographs of one representative

mouse from each group are shown over the time 10 weeks post adoptive transfer (A). Analysis of MusPV1 E6/E7 transcription in representative tails harvested from mice that had 10 weeks prior received by adoptive transfer either MusPV E6-specific CD8<sup>+</sup> T cells (B) or OT-1 T cells (C). Schematic of MusPV infection of RAG knock-out mice and subsequent adoptive transfer of MusPVE6 T-cell line or OT-1 cells as a control (D). Spleens were harvested from mice that had 10 weeks prior received by adoptive transfer either MusPV E6-specific CD8<sup>+</sup> T cells or OT-1 T cells. A flow cytometric analysis was performed after intracellular cytokine staining of these splenocytes for interferon- $\gamma$  and CD8 after stimulation with either mE6 or OVA peptide (E).

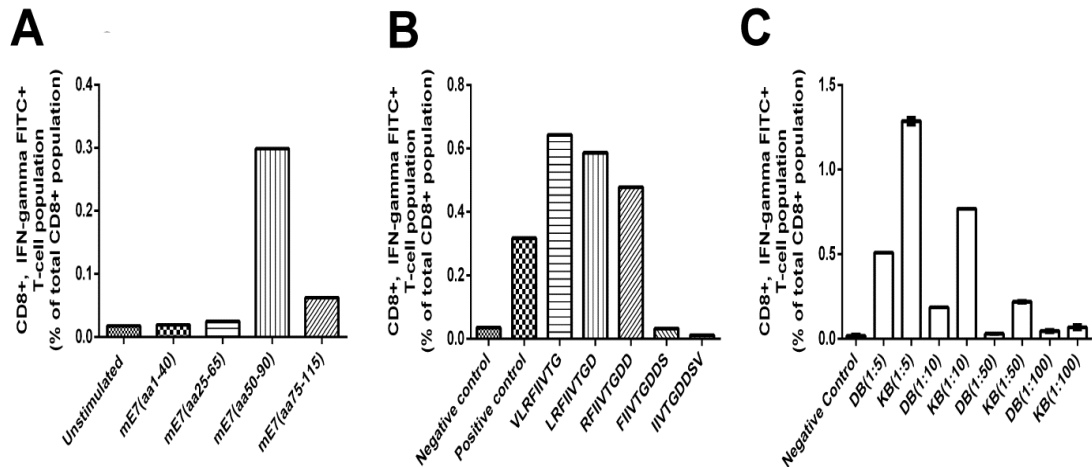




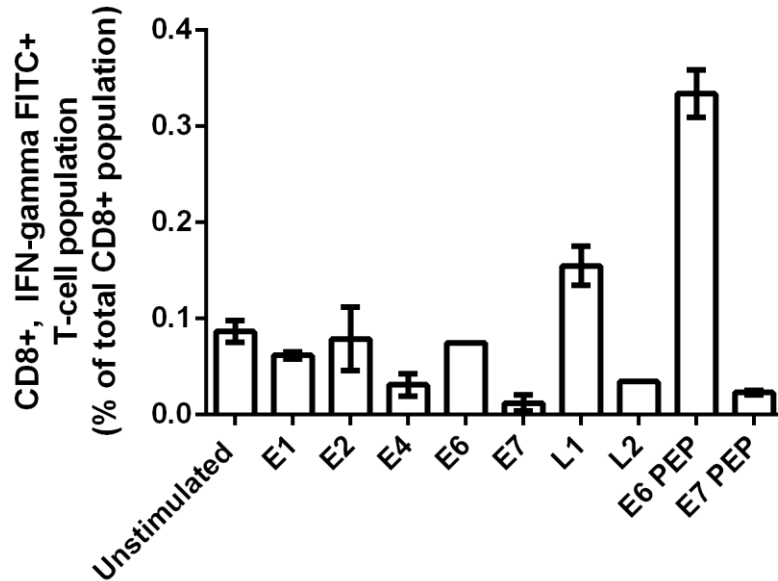
**Figure 36.** CD3 depletion allows the growth of papillomas on C57/BL6 mice (A) and Balb/c (B) therefore showing control of papillomavirus is due to T-cell immunity regardless of strain.



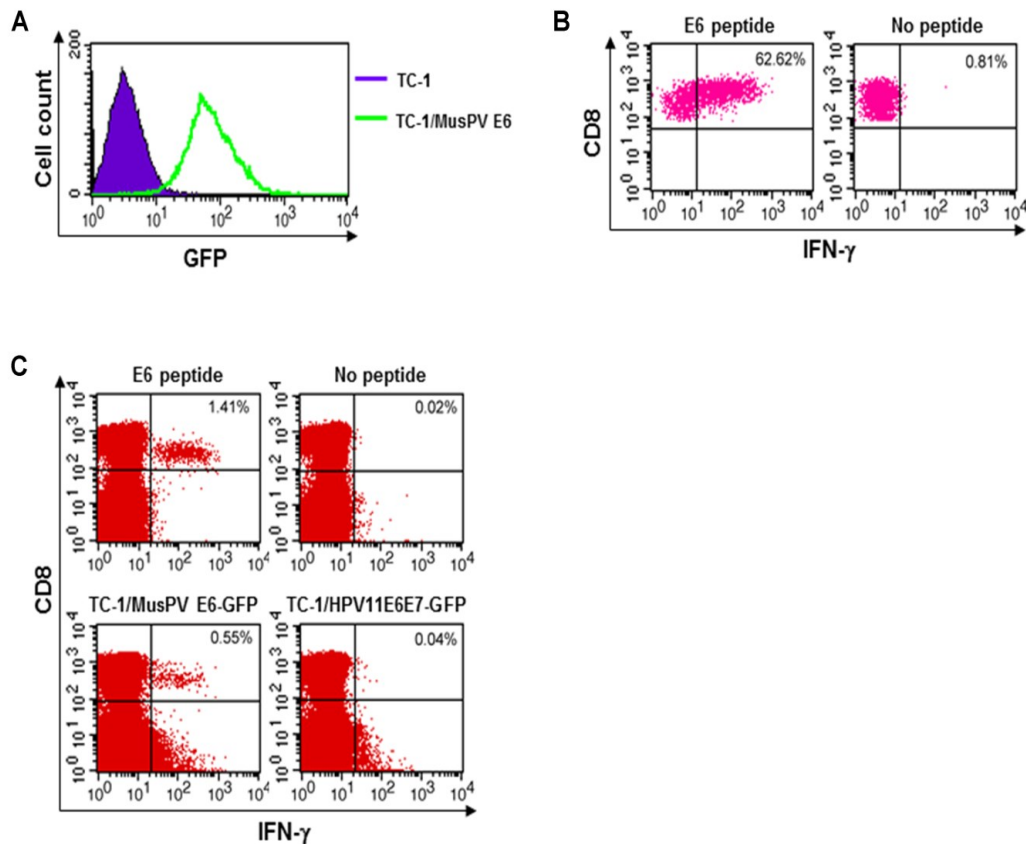
**Figure 37. Restoration of T-cell immunity results in wart regression.** CD3 depletion of n=9 Balb/c mice and the appearance of papillomas on their tail 5-weeks post infection. Following 5 weeks, mice were divided into group of 5 and 4 mice whereby the former continued undergoing CD3-depletion for another 5 weeks resulting in more obvious papillomas (B) whereas the remaining 4 whereby depletion was stopped resulted in wart regression.



**Figure 38. Determining the T-cell epitope of MusPV1 E7 and its MHC class 1 binding restriction via intracellular cytokine staining and flow cytometry analysis.** Bar graph of flow cytometry results after intracellular cytokine staining of splenocytes for interferon- $\gamma$  and CD8 after harvest from CRTmE7-vaccinated mice and stimulation with mE7 peptide library pools (A). Bar graph summarizing flow cytometry data after intracellular cytokine staining of splenocytes for interferon- $\gamma$  and CD8 after harvest from CRTmE7-vaccinated mice and stimulated with candidate 9mer peptides to map the immune-dominant MHC class I epitopes of MusPV1 mE7 (B). Bar graph showing flow cytometry data showing percentages of interferon- $\gamma$  expressing mE7 specific CD8+ T cells co-incubated with varying amounts of 293 cells expressing either the Murine MHC class 1 molecule H-2K<sup>b</sup> (293- K<sup>b</sup> or 293- K<sup>d</sup>) that were pulsed with the MusPV E7, immunodominant peptide, VLRFIIVTG to determine the MHC restriction. The results show that mE7 is K<sup>b</sup>-restricted (C).



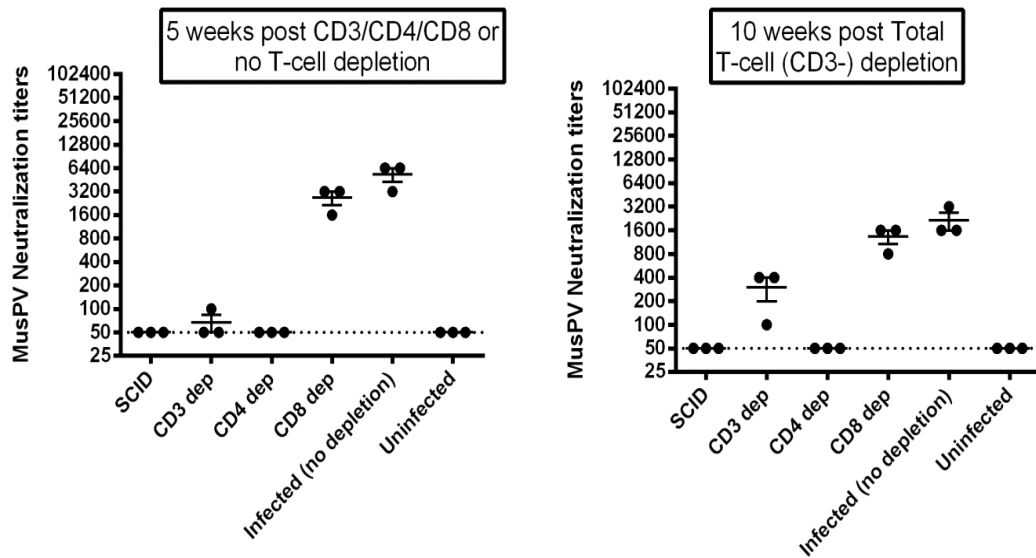
**Figure 39. Natural response to MusPV1-induced papilloma regression in C57/BL6 is mE6 dominant.** CD3-depletion of a group of 5 C57/BL6 bearing florid MusPV1 papilloma was stopped. Once the papilloma were completely regressed, splenocytes were harvested, pooled and incubated with 293<sup>Dbk<sup>b</sup></sup> cells which over-express the C57/BL6 MHC class I molecules were transfected with expression vectors for either hCRT-alone, or hCRT-linked to either MusPV1 E1, E2, E4, E6, E7, L1 or L2 respectively. The splenocytes were also stimulated directly using the MusPV1 E6 a.a. 90-99 (KNIVFVTVR) and E7 a.a. 69-77 (VLRFIIVTG) peptide epitopes. A specific mE6 specific CD8 T cell response could be detected in the splenocytes of C57/BL6 mice that had spontaneously cleared their papilloma only in the mE6 peptide stimulated cells.



**Figure 40. Establishment of murine MusPV E6-specific CD8<sup>+</sup> T cell line.** TC-1 cells were infected with lentiviruses expressing MusPV E6 and GFP, to establish MusPV E6-expressing TC-1 cells. GFP expressing TC-1 cells were analysed with flow cytometry analysis (A). TC-1 cells expressing MusPV E6 are able to activate MusPV E6-specific CD8<sup>+</sup> T cells after DNA vaccination. C57BL/6 mice were vaccinated with pcDNA3-CRT/MusPV E6 via intramuscular injection followed by electroporation. Splenocytes were prepared and stimulated with either MusPV E6 peptide, or irradiated TC-1/MusPV E6 or TC-1/HPV11E6E7GFP cells (control) at the presence of GolgiPlug overnight. The cells were stained with anti-mouse CD8 antibody. After permeabilization and fixation, the cells were stained anti-mouse IFN- $\gamma$  antibody. The cells were

acquired with FACSCalibur flow cytometer and analyzed with CellQuest Pro software (B).

Establishment of murine MusPV E6-specific CD8<sup>+</sup> T cell line. pcDNA3-CRT/MusPV E6 vaccinated C57BL/6 mouse splenocytes were stimulated with irradiated TC-1/MusPV E6 cells at the presence of murine IL-2 (20 IU/ml) once a week for four weeks. The cells were stimulated with MusPV E6 peptide at the presence of GolgiPlug overnight. IFN- $\gamma$  intracellular stained were then performed to determine the specificity of the CD8<sup>+</sup> T cells (C).



**Figure 41. CD4 T-cells are important for producing a viable anti-MusPV1 L1 antibody response.** Mice that were first depleted of CD3, CD4- or CD8- T-cell population were challenged with MusPV1 virus and assessed for their anti-L1 antibody titers 5 weeks post infection (A). MusPV1 infected mice that were first depleted of CD3, CD4- or CD8- T-cell population for 5 weeks, underwent 10 more weeks of CD3-depletion to fully immunosuppress their T-cell population.

Table 14: Summary of disease outcome following MusPV1 challenge of mice with respect to strain, genetic knock out and immune depletion.

<b>Mouse Strain</b>	<b>Immune treatment</b>	<b>Immune Status</b>	<b>Warts observed</b>
Nude Mice Outbred NCR(nu/nu)	N/A	No T cells	100% of all cases
SCID mice (BALB/C)	N/A	No B- and T cells	100% of all cases
BALB/C	Anti-CD3	No T cells	100% of all cases
BALB/C	Anti-CD4	No CD4 T cell population	0% of all cases
BALB/C	Anti-CD8	No CD8 T cell population	0% of all cases
BALB/C	N/A	Immunity	0% of all cases
C57/BL6	Anti-CD3	No T cells	0% of all cases
C57/BL6	Anti-CD4	No CD4 T cell population	0% of all cases
C57/BL6	Anti-CD8	No CD8 T cell population	0% of all cases
C57/BL6	N/A	Immunity	0% of all cases
C57/BL6	CD4-Knockout	No CD4 T cell population	0% of all cases
C57/BL6	CD40 Ligand- Knockout	In-active B-cells	0% of all cases
C57/BL6	Type-1 Interferon Knock-out	Decreased Anti-viral innate immunity	0% of all cases
SKH-1 Hairless mice (Outbred)	N/A	Immunity	15% of all cases (n=9/60)



**Table 15. Design of primers to amplify the different MusPV1 viral genes**

MusPV protein	Primer Set
E1	Forward 5'- GCGCGAATTCATGGAAAACGATAAAGGTACAGGG Reverse 5'- GAGAGCGGCCGCTTACTGCCTTTCTCGTAAAGG
E2	Forward 5' -GCGCGAATTCATGAACAGCCTGGAAACACGTTT -3' Reverse 5'- GAGAGCGGCCGCTCAGAGTCCGTCTAAGAAG
E4	Forward 5'- GCGCGAATTCATGAATCACCCCTTGGCTCCGAG Reverse 5'- GAGAGCGGCCGCTCACAGTCCAGTGAGAATAAT
E6	Forward 5'-GAGAAAGCTTCCACCATGGAGATCGGAAAAGGGTATA Reverse 5'- GACCGAGAATTCTTATCACAGCAGGGGTTTACAAAAG
E7	Forward 5'- GAGAAAGCTTCCACCATGCAGGGGCCTCTC Reverse 5'-GACCGAGAATTCTTATCACCTTTTCCATTCCGCAGATTC

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### **C. Concluding remarks and perspectives**

From a public health perspective, the introduction of Merck's nonavalent HPV vaccine is unlikely to resolve all problems associated with HPV disease. Since it is licensed for prevention of only 7 of the 15, albeit most common hrHPV [5], PAP screening should continue, thereby increasing rather than lessening the burden on current healthcare systems. Further, there is the problem of established HPV disease and high cost limiting access to the countries that need it the most. Presently, numerous efforts to develop generic HPV L1 VLP vaccines and L2-based approaches intended for local production at low cost to broaden access to this vaccine presents an important opportunity for cancer prevention across the globe and to better reach under-served populations. As mentioned, there are several L2-vaccines nearing phase 1 clinical trials and the work discussed in Chapters 1-3 in this thesis will potentially be useful to facilitate these upcoming vaccine trials by providing a robust, sensitive and high throughput methodology to measure the relevant immune correlate of protection, i.e. neutralizing IgG. Additionally, given the higher sensitivity of these assays and the validation work with human sera, it might be worth revisiting several L2-based HPV vaccines that were discounted due to low neutralization capabilities when tested with the conventional HPV neutralization assay.

With the respect to immune-therapy, much remains to be understood. Patients immune-suppressed patients by genetics, drugs for transplantation, or HIV co-infection, suffer HPV-associated cancers at a higher frequency than immune-competent individuals who clear HPV infection without apparent disease thus suggesting HPV infection can be controlled efficiently by an intact immune-system. However, as disease progresses, regressions become rarer, suggesting challenges to immunotherapy of cancer and greater burdens of high grade neoplasia. Here, in the final chapter of this thesis (Chapter 5), we attempted to examine mechanisms of immune clearance of papilloma produced by mouse papillomavirus (MusPV1/MmuPV1) in an out-bred immune-competent SKH-1 mouse model. Notably, a subset of the outbred SKH-1 mice were

genetically susceptible and developed persistent papilloma, whereas the remainder cleared their disease in time or never developed papillomas. An understanding of the genetic factors controlling disease outcomes in these mice might also help understanding of why patients (an outbred population) experience different clinical outcomes from the same HPV infection. Importantly, the ability to identify those that will clear the infection without intervention, and those needing interventions would be valuable clinically. Another important finding is that rejection of infection and disease requires both arms of T cell immunity, and in particular the importance of CD4 T cell responses. In C57/BL6 mice, the immunodominant CD8 T-cell epitopes were mapped within MusPV1 E6 and E7. A MusPV1 E6-specific effector CD8 T-cell line was generated from vaccination and upon systemic administration these T cells were able to traffic to the site of MusPV1 infection/disease and effect papilloma clearance. These findings highlight the promise of immunotherapy against papillomavirus infection and disease. However, a limitation of this study was that C57/BL6 is an in-bred strain. Studies with the SKH-1 mice with MusPV1 represents a promising model in which to evaluate candidate therapeutic vaccines.

On a broader scale, efforts to simplify delivery of HPV vaccines must continue, including examining co-delivery of HPV VLP vaccines with vaccines for other agents, and whether HPV vaccination could be initiated with childhood vaccination if the L2 antigen is included. A careful consideration of the impact of HPV vaccination on screening also must be made with respect to cost benefit, and whether the consequent reduction in the prevalence of cervical neoplasia renders the Pap smear insufficiently predictive as a first line screening tool. Another critical issue to address is whether HPV VLP vaccines protect men and women from HPV infection in the oral cavity, and thus from developing HPV-associated head and neck cancers. Currently there is early evidence to support this possibility [22] and rates of HPV-associated head and neck cancers should be followed in phase 4 vaccine studies in men and women.

In conclusion, it is anticipated that further significant advances will occur with respect to broadening protection to all hrHPV, lowering the number of doses and cost of HPV vaccination and eliciting therapeutic immunity. Such developments especially in immunotherapy of HPV will complement major advances in screening, notably HPV DNA, RNA and possibly oncoprotein testing as a first line screening modality in the cervix and also possibly at non-cervical sites. However, the advent of such technologies requires significant changes in health policies for best implementation and realization of their potential to eliminate HPV-related cancer and drive down costs so that all may benefit.

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## E. Curriculum Vitae

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## SUMMARY OF QUALIFICATIONS

Entrepreneurial microbiologist with a track record of research excellence in virology, immunology and vaccine development. Experienced in pre-clinical vaccine design and evaluation, specifically inventing, optimizing and operating immunologic antibody standards, cell-based immune-assays and mouse models to evaluate both antibody and T-cell responses. Successfully led or participated in collaborative scientific or business orientated teams that resulted in both high quality research data and successful research funding for science commercialization.

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## EDUCATION

2010- Present **Johns Hopkins University School of Medicine, USA**  
PhD Pathobiology (Expected March 2015)

2007-2010 **Imperial College London, United Kingdom**  
Bachelor of Science with Honours (First Class), Biology

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## RELEVANT SCIENTIFIC & BUSINESS MANAGEMENT SKILLS

- Vaccine Design & Development
- Epitope Mapping
- Immuno-Assay Development
- Fund Raising/Valuations
- Antibody Engineering
- *In Vivo* Imaging
- Risk Management
- Scientific/Grant/Business plan writing
- Flow Cytometry
- Animal Model Development
- Entrepreneurship
- Project Management

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## RESEARCH EXPERIENCE

2011-Present **PhD Candidate, Laboratory of Dr Richard Roden, Johns Hopkins Medicine**

- **Thesis Project: Preparation of 2<sup>nd</sup> generation HPV vaccines for Phase 1 Clinical Studies**
  - Focused on 2<sup>nd</sup> generation HPV vaccine development (both pre-clinical and clinical)
  - Engineered/Developed standards, cell-based assays or models to evaluate vaccine efficacy for Phase 1 clinical trial studies. Specifically:
    - A new sensitive cell-based antibody neutralization assay for HPV vaccines
    - Two human chimeric antibody standard for papillomavirus L2 minor capsid protein.
    - Evaluated a laboratory mouse papillomavirus model for HPV vaccine studies
  - These technologies have also been successfully transferred to several HPV research groups worldwide.
  - Secondary research topics include biological mechanisms of HPV L2 antibody neutralization and assessment of therapeutic HPV DNA vaccines and mouse models.
  - Excellent publication record ([Google Scholar](#)).
  - Awarded several department and governmental awards as well as travel grants to present my research at international scientific meetings.

2010-2011 **FYP Student, Laboratory of Professor Wendy Barclay, Imperial College London**

- **Final Year Project: Inhibition of swine origin H1N1 pandemic influenza with natural remedies**

- Investigated the antiviral potential of two over-the-counter medication products Sambucol™ and Vick's First Defence™.
- Conducted several *in vitro* dosage-dependent assays using cell-based systems to assess drug efficiency
- Acquired skills in various basic virology research techniques such as Plaque assays, MTT cell-proliferation assays and Haemagglutinin-inhibition assays

2009 **Summer Internship, Laboratory of Dr Robert Coutts, Imperial College London**

- **Project: Complete the genomic sequences of an unclassified mycovirus of the fungus *Phlebiopsis gigantea* and *Aspergillus fumigatus*.**
  - Successful in obtaining a student bursary from the British society of plant pathology (BSPP) to finance this project.
  - Screened a worldwide panel of *Aspergillus fumigates* and *Phlebiopsis gigantean* isolates for the presence of double-stranded RNA mycoviruses (viruses that infect fungi) and investigated if these elements are ubiquitous in their respective fungi.
  - Characterise the sequences of both these dsRNA elements using a combination of genome-walking techniques and RT-PCR extension reactions.

2009 **Summer Internship, Laboratory of Lisa Ng Fong Poh, Singapore Immunology Network**

- **Project: Understanding innate and adaptive immune responses against *Chikungunya* virus infection via virus-like particles (VLP).**
  - Acquired proper understanding and basic skills in general molecular biology, cell culture and DNA recombinant technology techniques.

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**BUSINESS, MANAGEMENT AND PROFESSIONAL DEVELOPMENT**

2011- Present **Co-Founder, PathoVax LLC**

- Co-founded PathoVax LLC, a Johns Hopkins Biotechnology Start-up
- Major achievements/milestones
  - Securing \$6000 and winning the "Local Medical Product category" at the 'A Call for Innovation' competition during the 2014 JHU Entrepreneurship week.
  - Securing \$10,000 for Innovation & Capability Voucher Grant to support Patent Landscape and Freedom to Operate Analysis for PathoVax's propriety product

2007- 2010 **Student Leader in various organizations, Imperial College London**

- **Biology Third Year Department Representative (2009/2010)**
  - Elected as a spokesperson for 3<sup>rd</sup> year undergraduate students in the Biology department
  - Responsible for keeping both students and staff abreast on issues related to activities, academics and welfare
- **Vice-President of Imperial College Singapore Society (ICSS) (2008/09)**
  - Fostered strategic partnerships with both governmental (Contact Singapore, Overseas Singaporeans,) and private companies/organizations (Accenture, GNC) for funding.
  - Secured over £1500 pounds in sponsorships for the society's financial year of 2008/09.
  - Co-producer of "Ah Yum", an annual Singaporean Musical charity event where all proceeds (£2000) pounds were donated to charity (*Children's Cancer and Leukaemia Group*, United Kingdom)

2007 **Bank Sales Officer, Citi Singapore Ltd/ CPP Sales/Acquisitions Department**

- Involved in credit card sales.
- Encourage customers to enrol for Citibank's credit cards and biometric credit card services



- Addressed customer's needs and queries.
- Able to exceed required quota of credit card and bio-metric enrolment sales target of 500 customers per month

**2003-2006 Infantry Officer, Singapore Armed Forces (Rank: 2<sup>nd</sup> Lieutenant)**

- Responsible for the training, discipline and welfare of the Manpower and Regimental Police Operations branch.
- Addressed servicemen's needs and concerns monthly
- Conducted recruitment sessions on a quarter yearly basis for servicemen who were interested in a career in the Singapore Armed Forces.
- Conducting Officer for the Advanced Train-fire Program (ATP), a quarterly live-firing exercise and helped the unit achieved passing rate of 92% (highest in the year of 2005 for the unit).
- Finished my military service with the highest honors of 'Outstanding'

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## CERTIFICATIONS

### **The Wharton School Business Foundations Specialization Series**

- Financial Accounting
- Marketing
- Corporate Finance
- Operations Management

Coursera & The Wharton School, University of Pennsylvania (Ongoing)

### **Managing Science in the BioTech Industry**

American Society for Cell Biology & Keck Graduate Institute, 2014

### **Medical Entrepreneurship: Bridging the Gap between Research and Practise**

Medical & Educational Perspectives, Inc, 2013

### **Clinical Vaccine Trials and Good Clinical Practice**

The Johns Hopkins Bloomberg School of Public Health, 2012

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## PUBLICATIONS

### **Published Manuscripts**

- 1) Peng S, **Wang JW**, Karanam B, Wang CG, Hung CF, Wu TC, Huh WK and Roden RB (2014) Enhancement of antitumor immunity after cisplatin treatment by formulation of HPV16 E6E7L2 fusion protein with GPI-0100 adjuvant and intra-tumoral vaccination. *PLOS One* 10.1371/journal.pone.0116389
- 2) **Wang JW**, CF Hung, Huh WK, Trimble CL and Roden RB (2014) Immunoprevention of human papillomavirus-associated malignancies *Cancer Prevention Research canprevres*. 0311.2014
- 3) **Wang JW**, Jagu S, Wang C, Kitchener HC, Daayana S, Stern PL, Pang S, Day PM, Huh WK, Roden RB (2014) Measurement of neutralizing serum antibodies of patients vaccinated with human papillomavirus L1 or L2-based immunogens using furin-cleaved HPV Pseudovirions. *PLoS One*. 9(7):e101576.

- 4) Kwak K, Jiang R, **Wang JW**, Jagu S, Kirnbauer R, Roden RB (2014) Impact of inhibitors and L2 antibodies upon the infectivity of diverse alpha and beta human papillomavirus types. *PLoS One*. 9(5):e97232
- 5) Peng S, Song L, Knoff J, **Wang JW**, Chang YN, Hannaman D, Wu TC, Alvarez RD, Roden RB and Hung CF (2014). Control of HPV-associated tumors by innovative therapeutic HPV DNA vaccine in the absence of CD4+ T cells. *Cell Bioscience*. 4(1):11
- 6) **Wang JW**, Jagu S, Kwak K, Wang C, Peng S, Kirnbauer R, Roden RB (2014) Preparation and properties of a papillomavirus infectious intermediate and its utility for neutralization studies *Virology*. 449:304-16.
- 7) Anchoori RK, Karanam B, Peng S, **Wang JW**, Jiang R, Tanno T, Orłowski RZ, Matsui W, Zhao M, Rudek MA, Hung CF, Chen X, Walters KJ, Roden RB. A bis-benzylidene piperidone targeting proteasome ubiquitin receptor RPN13/ADRM1 as a therapy for cancer. *Cancer Cell*. 2013;24(6):791-805.
- 8) **Wang JW** and Roden RB. (2013) L2, the minor capsid protein of papillomavirus. *Virology*. 2013 May 17. pii: S0042-6822(13)00226-2. doi: 10.1016/j.virol.2013.04.017.
- 9) Kwak K, Jiang R, Jagu S, **Wang JW**, Wang C, Christensen ND, Roden RB. (2013) Multivalent human papillomavirus 11 DNA vaccination utilizing electroporation. *PLoS One*. 2013;8(3)
- 10) **Wang JW**, Roden RB. (2013) Virus-like particles for the prevention of human papillomavirus-associated malignancies. *Expert Rev Vaccines*. 2013 Feb;12(2):129-41

#### Manuscripts submitted/In-Press

1. **Wang JW**, Matsui K, Peng S, Kemp T, Pinto LG and Roden RB (2015) Production of Furin-cleaved Papillomavirus Pseudovirions and their use for *in vitro* neutralization assays of L1 or L2-specific antibodies (*Current Protocols in Microbiology*, *In Press*)
2. **Wang JW**, Jagu S, Wu WH, Viscidi RP, Macgregor-Da, A, Fogel JM, Kwak K, Daayana Sai, Kitchener HC, Stern PL, Gravitt P, Trimble CL and Roden RB (2014) Human Chimeric papillomavirus L2-specific antibodies (*Clinical and Vaccine Immunology (CVI)*, *In Press*)
3. **Wang JW\*\***, Jiang R\*\*, Peng S, Hung CF and Roden RB. Natural and Vaccinated Immune characteristics of a Laboratory Mouse Papillomavirus Model (MusPV) (\*\* indicates authors contributed equally to the work) (Manuscript pending)
4. Wu WH\*\* **Wang JW\*\***, Kwak K, Hung CF and Roden RB. Roles of Fc domain and exudation in L2 antibody-mediated protection against human papillomavirus (\*\* indicates authors contributed equally to the work) (Manuscript pending)

**AWARDS/SCHOLARSHIP/BURSARY RECEIVED**

<b>Type of Award</b>	<b>Amount</b>	<b>Year</b>
<p><b>Local Medical Product Award, Johns Hopkins Entrepreneurship Week 2014</b></p> <p>Chief Scientific Officer of the winning team (PathoVax) that won the Local Medical Product category at the 'A Call for Innovation' competition during the 2014 JHU Entrepreneurship week. The entire PathoVax was awarded a shared cash prize (\$1000), in addition to financial support of up to \$5000 and further mentorship to develop and commercialize the team's winning idea.</p>	\$6000 USD	2014
<p><b>2014 Pathology Young Investigators Day Award for Excellence in Translational Research</b></p> <p>Received an award for excellence in translational research from the Department of Pathology for my research in developing a new human chimeric monoclonal antibodies specific for Human papillomavirus minor capsid protein L2</p>	\$500 USD	2014
<p><b>2013 Pathology Young Investigators Day Award for Excellence in Translational Research</b></p> <p>Received an award for excellence in translational research from the Department of Pathology for my research in developing a new sensitive human papillomavirus in vitro neutralization assay during the 15<sup>th</sup> Annual Young Investigators' Day at Johns Hopkins University</p>	\$500 USD	2013
<p><b>Contact Singapore-Global Young Scientist Summit Singapore 2013 Travel Award</b></p> <p>Applied and received a travel award to attend the inaugural GYSS@one-north to be held in Singapore from 20<sup>th</sup>-25<sup>th</sup> January 2013)</p>	\$1000 USD	2012
<p><b>NIH Travel Award</b></p> <p>Applied and received a travel award for the 28<sup>th</sup> International Papillomavirus Conference in San Juan, Puerto Rico)</p>	\$1000 USD	2012
<p><b>Johns Hopkins School of Medicine Graduate Student Association Travel Award</b></p> <p>Applied and received a travel award for 27<sup>th</sup> International Papillomavirus Conference in Berlin, Germany)</p>	\$100 USD	2011
<p><b>Johns Hopkins School of Public Health Global Health Established Field Placement Award</b></p> <p>Awarded grant to participate in HIV behavioural research in Chiang Mai, Thailand in the summer of 2011. The objective of this award is to enhance the recruitment of students into global health research and practice careers by providing them the means to work with global health mentors and to attain international cross-cultural field experience.)</p> <p>For more information on this project, please visit: <a href="#">here</a></p>	\$3500 USD	2011
<p><b>Margaret Lee Fellowship</b></p>	\$27,000 USD	2010-

Scholarship for PhD studies at Johns Hopkins University. This includes full funding on stipend, health insurance and school fees	(Stipend)	Present
<b>British Science Festival Bursary</b> Awarded to top 5% of Biology undergraduates in department of Biology at Imperial College to participate in the 2009 British Science Festival. This bursary scheme offers an outstanding opportunity for students to broaden their horizons, meet leading scientists and speakers, engage in debate and discussion about the impact of science in society and enjoy the atmosphere of an event that is extensively covered in the media.)	£500 GBP	2009
<b>British Society of Plant Pathology Summer Vacation Student Bursary</b> Undergraduate bursary grant for 10 weeks to provide support for work on specified research projects during the summer vacation. For more information on this project, please visit: <a href="#">here</a> and read page 30	£2500 GBP (£200/week) and £500 to the lab for research consumables	2009
<b>Outstanding Performance for Singapore Armed Forces Military Service</b> Vocation: Infantry Officer Rank: Lieutenant	n/a	2003-2006