

## Short Communication

# Binding and neutralization characteristics of a panel of monoclonal antibodies to human papillomavirus 58

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Human papillomavirus (HPV) 58 is a high-risk HPV type associated with progression to invasive genital carcinomas. We developed six monoclonal antibodies (mAbs) against HPV58 L1 virus-like particles that bind conformational epitopes on HPV58. The hybridoma cell lines were adapted to serum- and animal component-free conditions and the mAb supernatants were affinity-purified. The six mAbs neutralized HPV58 pseudoviruses (PsVs) and 'quasivirions' with different capacities. The mAbs differed in their ability to prevent PsV58 attachment to HaCaT cells, to the extracellular matrix (ECM) deposited by HaCaT cells, to heparin and to purified human laminin 5, a protein in the ECM. These mAbs provide a unique set of tools to study the binding properties of a previously untested, high-risk HPV type and the opportunity to compare these characteristics with the binding of other HPV types.

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Human papillomaviruses (HPVs) are small, non-enveloped viruses with an 8 kb double-stranded DNA genome encapsidated in a structure consisting of 72 capsomers composed of pentameric L1 protein. There is also a minor capsid protein, L2, that has not been characterized fully (Kirnbauer *et al.*, 1993). The recent vaccines Gardasil and Cervarix, approved for use in humans, include L1 virus-like particle (VLP) antigens of the high-risk HPV types for cervical cancer, HPV16 and 18, and Gardasil also includes the low-risk HPV types 6 and 11 (Harper *et al.*, 2006; Schmiedeskamp & Kockler, 2006; Stanley, 2006; Villa *et al.*, 2005). HPV types 31, 33, 39, 45, 52 and 58 are the next most frequently detected genotypes in cervical cancers after HPV16 and 18 (Munoz *et al.*, 2003). HPV58 is considered worldwide to be a rare type, but is prevalent in cervical cancers in eastern Asia (Chan *et al.*, 2002). In Mexico, HPV58 was found in all grades of lesions at a frequency equal to or higher than HPV16 (Gonzalez-Losa *et al.*, 2004). Several researchers have indicated that HPV58 needs to be included in a second-generation HPV vaccine in order to provide better protection in Asia and Africa (Bhatla *et al.*, 2008; Fukuchi *et al.*, 2009; Xi *et al.*, 2003). To date, there are no published monoclonal antibody (mAb) reagents to HPV58, making study of the binding and neutralization properties of this virus type difficult.

Previously, we demonstrated that HPV11 virion infection is blocked by varying degrees of epitope saturation by HPV11-

specific mAbs (Christensen *et al.*, 1994). Neutralization of HPV11 virions is also achieved by several monovalent Fab fragments and single-chain variable fragments (scFv) (Culp *et al.*, 2007). It was reported that mAbs to HPV16 (H16.V5, H16.E70 and H16.U4) differ in the mechanisms utilized to neutralize HPV16 pseudoviruses (PsVs) (Day *et al.*, 2007). V5 and E70 did not prevent attachment of the capsids to the cell surface, but did block their binding to the extracellular matrix (ECM). U4 blocked binding to the cell surface, but not to the ECM.

Most reports describe studies that are conducted with HPV11, 16 or 31 and there are few data comparing the binding patterns of capsids and virions of different HPV types to human keratinocytes and to the ECM secreted by these cells (Culp *et al.*, 2006b; Day *et al.*, 2003; Selinka *et al.*, 2007; Smith *et al.*, 2007). A recent publication compares HPV16, 31 and 5 PsVs binding to the murine genital tract (Johnson *et al.*, 2009). Data from our laboratory demonstrate that there are differences in binding properties between VLPs of HPV11, 16, 18 and 45 to HaCaT cells and ECM, demonstrating that it is important to study several different HPV types to determine binding and uptake differences and similarities *in vitro* and ultimately *in vivo* (Broutian *et al.*, 2010). Type-specific reagents, including mAbs, are needed to study properties of individual HPV types, as cross-reactivity is limited among mAbs (Rizk *et al.*, 2008).

In the current study, we developed six type-specific and neutralizing HPV58 mAbs and determined their binding and neutralization titres. We then tested the ability of the mAbs to inhibit the binding of PsV58 to heparin–BSA and purified human laminin 5 (LN5) and to HaCaT cells and ECM. These mAbs will be useful tools in determining the neutralizing epitopes on HPV58 capsids and comparing the binding and entry mechanisms of HPV58 with those of other HPV types.

HPV58 L1 VLPs and pseudovirions (L1 and L2 encapsidating a pYSEAP genome) were prepared as described previously (Buck *et al.*, 2005; Pastrana *et al.*, 2004). ‘Quasivirions’ (QVs), a term coined by our laboratory to describe virions with an authentic papillomavirus genome produced in 293TT cells, were prepared as described previously (Mejia *et al.*, 2006; Pyeon *et al.*, 2005). For this study, HPV58 L1 and L2 encapsidate an HPV11 genome. Hybridomas secreting HPV58 L1-specific mAbs were generated as described previously using Ribi adjuvant (Corixa) (Christensen *et al.*, 1990, 1996). Hybridoma cell lines were adapted to serum-free conditions in animal component-free media (BD Biosciences) and supernatants were purified on Protein A affinity columns for all IgG mAbs. The single IgM mAb was purified on an immobilized mannan-binding protein column (Pierce). mAb protein concentrations were determined by  $A_{280}$  readings. PsV, VLP and QV protein concentrations were determined by BCA protein assay (Pierce). Approximately  $9.75 \times 10^9$  VLPs or PsV particles were used in ELISA binding assays to determine the mAbs’ reactivity against HPV58 PsVs and L1 VLPs [Christensen *et al.*, 1996; Schiller laboratory technical file 129 (<http://ccr.cancer.gov/staff/links.asp?profileid=5637>)].

Neutralization assays with PsV58 were performed in 293TT cells as described previously (Buck *et al.*, 2005; Pastrana *et al.*, 2004). Approximately  $1.6 \times 10^5$  PsVs per cell were incubated with indicated dilutions of mAbs for 1 h at 37 °C before adding to duplicate wells. Two days post-seeding, 30  $\mu$ l cell-culture supernatant was assayed with pNPP (Sigma). Neutralization of QVs was performed in HaCaT cells and 293TT cells, where approximately  $1.38 \times 10^6$  QVs per cell were incubated with dilutions of mAbs before adding to cells. Seventy-two hours post-seeding, cells were harvested with TRIzol (Invitrogen) and total RNA was extracted. E1<sup>^</sup>E4 transcripts were determined with quantitative (Q) RT-PCR and REST analysis as described previously (Culp & Christensen, 2003).

ELISA binding assays to heparin–BSA and LN5 were conducted as described previously (Culp *et al.*, 2006b) with minor modifications. Heparin–BSA- or mAb affinity column-purified human LN5 (200 ng per well) was coated onto microtitre plates (Evergreen Scientific) overnight at 4 °C. PsVs were incubated overnight at 4 °C with 100 ng mAbs ml<sup>-1</sup> in PBS. Pre-incubated PsVs or PsVs alone were added to milk protein-blocked duplicate wells for 1 h at room temperature. After washing, a rabbit polyclonal

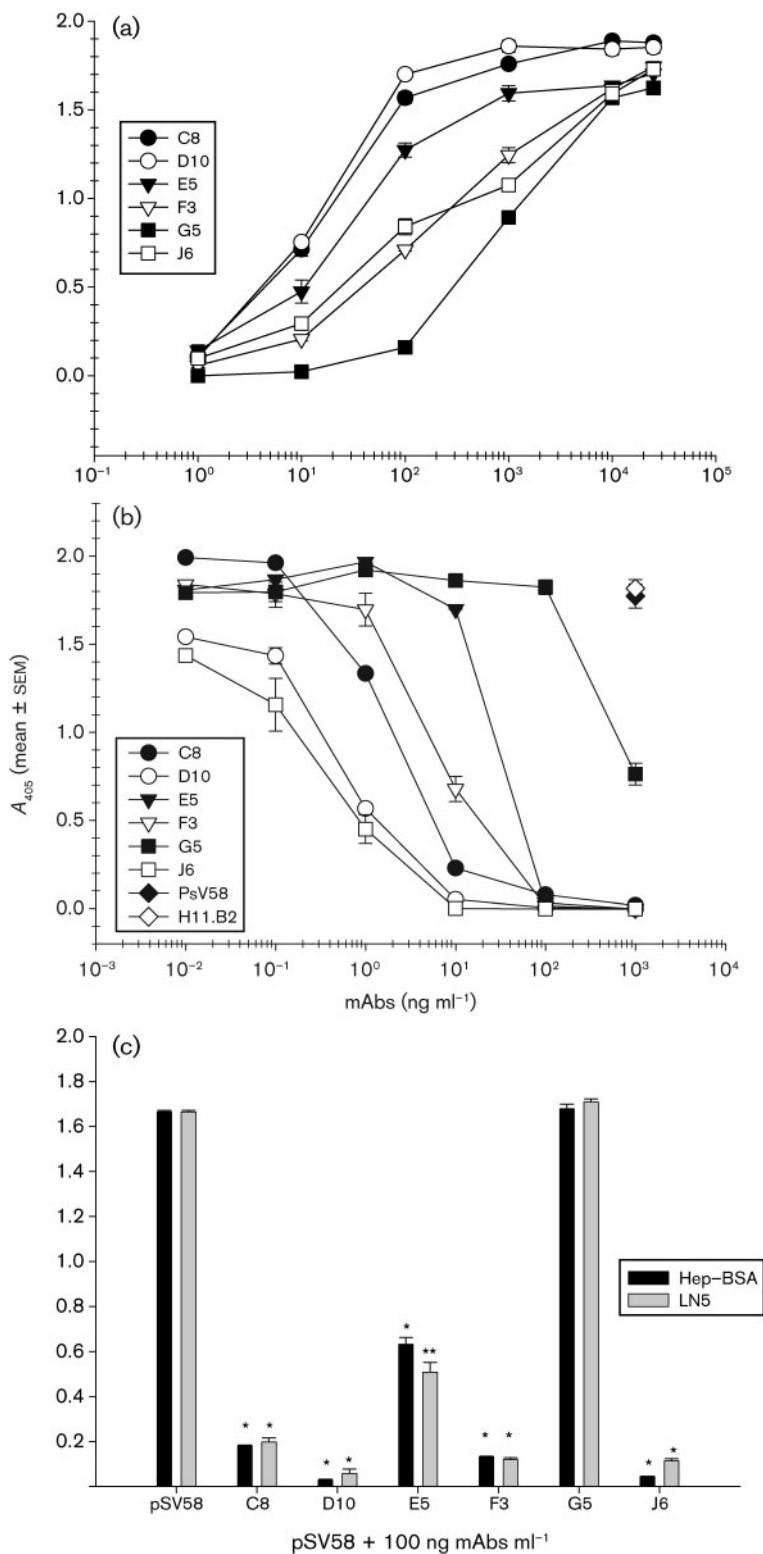
antibody raised against HPV58 L1 VLPs was added in 5 % milk PBS/T followed by an anti-rabbit secondary antibody (Pierce) conjugated to alkaline phosphatase.

Immunofluorescence studies of mAb inhibition of PsV58 binding to HaCaT cells and ECM were described previously (Culp *et al.*, 2006a). PsV58 (10  $\mu$ g ml<sup>-1</sup>) was incubated with 100 ng mAbs ml<sup>-1</sup> overnight at 4 °C, added to fixed HaCaT cells and ECM and detected by a pool of the H58 mAbs. Fluorophore-labelled secondary antibodies were goat anti-mouse–Alexa Fluor 488 IgG and donkey anti-rabbit–Alexa Fluor 594 (Invitrogen). All coverslips were stained with Hoechst 33342 (Molecular Probes) to detect cellular DNA.

Fluorescence microscopy was performed using a Nikon Eclipse E600. Photographs were digitally prepared using Adobe Photoshop. Within each figure, all images were photographed and digitally prepared in an identical manner.

Six reactive hybridoma clones were selected for further study: H58C.8 (C8), H58D10.6 (D10), H58E5.1 (E5), H58F3.1 (F3), H58G5.1 (G5) and H58J6.3 (J6). Binding titres were determined by ELISA using intact or denatured HPV58 L1 VLPs or intact HPV58 PsVs. All six mAbs detected a conformationally sensitive epitope of HPV58 L1 VLPs or PsVs, as none bound to denatured L1 VLPs (data not shown). The binding profiles of the mAbs to HPV58 PsVs are shown in Fig. 1(a). The strongest half-maximal binding titre for PsV58 was shown with mAb D10 (15 ng ml<sup>-1</sup>), whilst the lowest half-maximal titre was with mAb G5 (700 ng ml<sup>-1</sup>). The half-maximal binding titres to PsVs and L1 VLPs are shown in Table 1. The first mAb to reach maximum absorbance was G5 (absorbance 1.62). Interestingly, the absorbance for mAb J6 was only 0.29 at the same time point, despite having an apparent half-maximal binding titre of 100 ng ml<sup>-1</sup> (same time point data not shown). We wondered whether this indicated that J6 has fewer epitope-binding sites than the other mAbs. A capture ELISA was performed whereby unconjugated goat anti-mouse antibody was bound to ELISA wells in sodium carbonate pH 9.6 binding buffer, followed by dilutions of the mAbs and secondary antibody. mAb J6 showed the same detection pattern as in the PsV-binding ELISA, indicating that the secondary antibody may not detect IgG<sub>3</sub> isotypes efficiently and that we therefore cannot make conclusions about the number of epitope-binding sites from the indirect ELISA. The half-maximal binding titre for J6 is only an approximation, due to the inefficiency of detection by the secondary antibody.

The neutralizing activities of the mAbs to PsV58 in 293TT cells are shown in Fig. 1(b). The half-maximal neutralizing titres determined for each mAb are shown in Table 1. mAb J6 was the strongest neutralizer (0.2 ng ml<sup>-1</sup>). The strongest binder, mAb D10, was the second-strongest neutralizer at 0.4 ng ml<sup>-1</sup>. Not surprisingly, mAb G5 was the weakest neutralizer (690 ng ml<sup>-1</sup>). These data indicate that the approximate half-maximal binding titre for J6 may not be accurate, as the half-maximal neutralization titre is 500-fold lower than the binding titre for PsVs.



**Fig. 1.** (a) Binding titres of mAbs to HPV58 PsVs, (b) neutralization of PsV58 and (c) mAb inhibition of binding of PsVs to heparin-BSA or LN5 by ELISA-based binding or neutralization assays. One asterisk indicates a *P*-value of <0.001 and two asterisks indicate a *P*-value of <0.01. The absorbance is indicated on the *y*-axis and the dilution of antibody (a, b) or well treatment (c) is indicated on the *x*-axis. Each figure is representative of three separate experiments.

We also utilized QVs containing HPV58 capsids and an HPV11 genome to determine neutralization capacities in human keratinocyte (HaCaT) cells and in 293TT cells. 293TT cells do not secrete LN5 into their ECM (Culp *et al.*, 2006a), so HaCaT cells were utilized as a more suitable

host-cell line. Table 1 shows the half-maximal binding titres of three selected mAbs (D10, E5 and J6) as determined by QRT-PCR and REST analysis of E1<sup>^</sup>E4 transcripts in HaCaT cells and 293TT cells (Culp & Christensen, 2003; Mejia *et al.*, 2006). Interestingly, the



**Table 1.** Binding and neutralization characteristics of H58 mAbs and neutralization titres of QV58 in 293TT and HaCaT cells

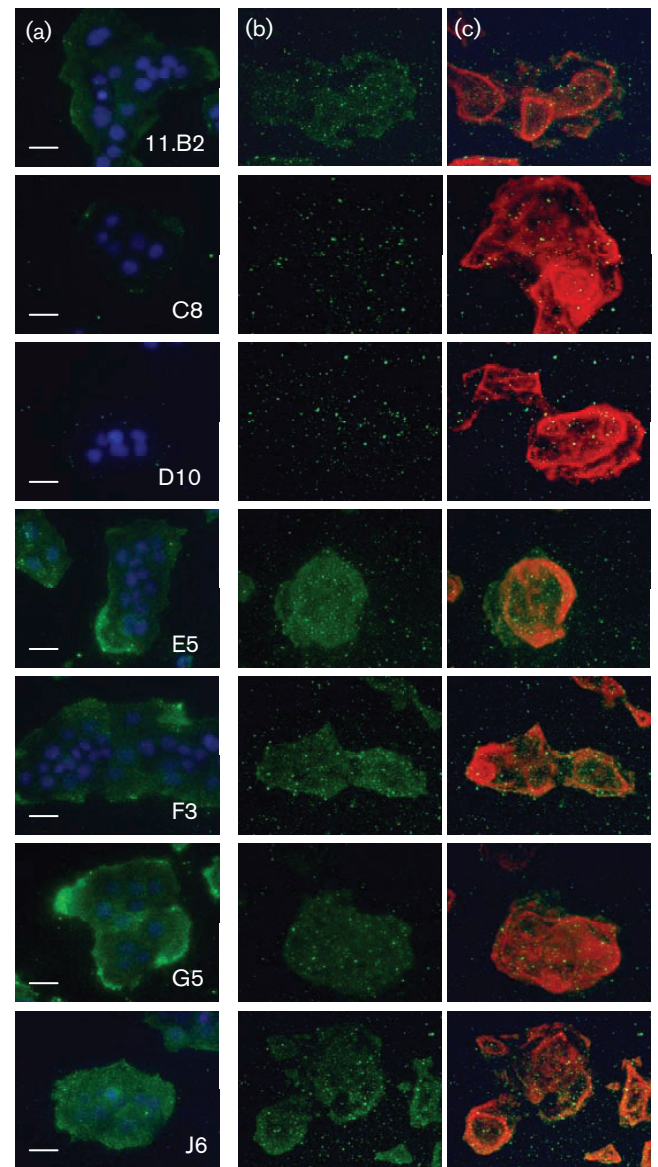
mAb	Isotype	Epitope	Binding titre (ng ml <sup>-1</sup> )		PsV58 neutralization titre (ng ml <sup>-1</sup> )	QV neutralization (ng ml <sup>-1</sup> ) in:	
			PsVs	VLPs		HaCaT cells	293TT cells
H58C8	IgG <sub>1</sub>	C	18	29	1.2	–	–
H58D10	IgG <sub>2a</sub>	C	15	15	0.4	48	125
H58E5	IgG <sub>2a</sub>	C	28	25	20	500	3800
H58F3	IgM	C	200	175	6	–	–
H58G5	IgG <sub>2b</sub>	C	700	2250	690	–	–
H58J6	IgG <sub>3</sub>	C	~100*	~1100*	0.2	400	600

\*Half-maximal binding titres for J6 are approximate due to inefficient secondary antibody detection.

neutralization titres were higher for 293TT cells for all three mAbs tested, although those for D10 and J6 were only 2-fold higher. The neutralization titre for mAb E5 was 7.5-fold higher. Also, the neutralization titres for QVs in both cell lines were much higher than that for PsVs in 293TT cells. This is probably a direct result of the higher capsid doses utilized for QVs in order to achieve sufficient E1<sup>Δ</sup>E4 transcripts and the different quantitative end points (E1<sup>Δ</sup>E4 transcripts versus secreted alkaline phosphatase and colorimetric readings). These data also indicate that HPV58 may infect by unique pathways, depending on the receptors available in the cells and/or ECM, but further studies need to be completed comparing different host-cell lines.

Next, we were interested in determining whether the mAbs could inhibit PsV58 binding to heparin–BSA- and/or purified human LN5-coated microtitre plates, as some studies have suggested that heparan sulfates play a role in virus–cell binding and infection (Combita *et al.*, 2001; Day *et al.*, 2007; Joyce *et al.*, 1999; Patterson *et al.*, 2005; Selinka *et al.*, 2007). In addition, our laboratory has shown that LN5 is important in HPV11 infection (Culp *et al.*, 2006b). H11.B2, a previously described mAb, was included as a negative control (Christensen *et al.*, 1990). The PsVs were incubated with 100 ng mAbs ml<sup>-1</sup> and detected by a rabbit polyclonal antibody (pAb) raised against HPV58 L1 VLPs (Rb58 pAb). We determined previously that Rb58 pAb can detect PsV58–mAb complexes bound to uncoated ELISA wells (data not shown). Fig. 1(c) shows that mAb G5 did not block binding to either heparin–BSA or LN5. C8, D10, F3 and J6 mAbs blocked binding to heparin–BSA and LN5 with *P*-values of <0.001, as determined by Student's *t*-test. mAb E5 blocked binding to heparin–BSA with a *P*-value of <0.001 and to LN5 with a *P*-value of <0.01.

Binding characteristics of PsV58 to HaCaT cells and ECM were examined by using the panel of mAbs as specific tools for capsid detection. First, we performed immunofluorescence assays, where it was determined that PsV58 binds to HaCaT cells and also to the ECM secreted by these cells. A pool of the mAbs at a total dilution of 1:1000 resulted in effective detection of PsV58 on cells and ECM (data not shown).



**Fig. 2.** mAb inhibition of PsV58 binding to HaCaT cells or HaCaT-derived ECM. (a) PsV58 binding to cells; (b) PsV58 binding to ECM; (c) merge of PsV58 and LN5 staining. This figure is representative of three separate experiments. Bars, 10  $\mu$ m.

mAbs have been useful probes to determine binding properties of VLPs and virions. PsV58 incubated with 100 ng mAbs ml<sup>-1</sup> was added to fixed HaCaT cell cultures or to HaCaT-derived ECM to test for binding inhibition. PsV58 incubated with H11.B2 was included for each experiment as a binding control (Fig. 2). A pAb to LN5 was included on ECM staining. These experiments showed several possible mechanisms for neutralization of PsV58 by the panel of mAbs. mAbs C8 and D10 blocked binding of PsV58 to HaCaT cells and ECM, as PsV58 binding was no longer visible under our detection conditions. The other four mAbs did not block binding of PsV58 to cells or to ECM at this dilution. Taken into consideration with the QV neutralization data in HaCaT cells, these findings indicate that mAbs C8 and D10 probably neutralize by inhibiting the PsV/QV from binding to host receptors on the HaCaT cells and ECM. The other mAbs appear to neutralize by a different mechanism, possibly by inhibiting internalization of the PsVs/QVs or by blocking binding to a potential secondary receptor.

All of the mAbs except G5 were able to block PsV58 binding to purified heparin-BSA and LN5 in an ELISA, but only mAbs C8 and D10 were able to block fully the binding to HaCaT cells and ECM at the same mAb concentration. The ELISA tested the mAbs' effectiveness at inhibiting the PsV from binding to only one purified receptor/component. HaCaT cells and ECM have multiple receptors available for PsV binding, including heparan sulfates, LN5 and  $\alpha 6$  integrin among others. Collectively, these observations confirm the complexity of the binding/neutralization processes and indicate the possibility that HPV capsids use several receptors on the cells and ECM for infection. Purified mAbs are excellent reagents to determine binding and neutralization titres, as all cell-culture contaminants have been removed. These mAbs will be useful tools in determining the cell surface/ECM-binding receptors of HPV58, for mapping the neutralizing epitopes and for determining the internalization pathway of another high-risk HPV type.

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