

# Targeting Pemphigus Autoantibodies through their Heavy-Chain Variable Region Genes

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Pemphigus vulgaris (PV) is a potentially fatal blistering disease characterized by autoantibodies against cell surface adhesion proteins desmoglein (Dsg) 3 and Dsg1. Previous studies using phage display to clone Dsg-reactive monoclonal antibodies from a PV patient demonstrated that a limited number of antibody variable region genes encode the autoantibody repertoire, with different genes for pathogenic and non-pathogenic mAbs. Here, we investigated the feasibility of specific autoantibody targeting in pemphigus. We produced rabbit anti-idiotypic antibodies against two pathogenic and two non-pathogenic PV mAbs. Antisera inhibited binding of the immunizing mAb to Dsgs by ELISA as well as pathogenicity against cultured human keratinocytes. Antisera also inhibited other mAbs using the same variable region heavy chain ( $V_H$ ) genes, despite different light chains or somatic mutations. Additionally, peptide phage display identified peptide sequences that bound PV mAbs in a  $V_H$ -specific manner. To evaluate the therapeutic potential of  $V_H$  gene-targeted reagents, preimmune sera and antisera were used to adsorb pathogenic antibodies from PV sera. Pooled antisera significantly reduced pathogenic activity from the original PV patient's serum and bound pathogenic antibodies from two other PV sera, suggesting shared autoantibody  $V_H$  gene usage among PV patients. Together, these data suggest novel  $V_H$  gene-targeted approaches toward PV treatment.

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## INTRODUCTION

Pemphigus is an autoimmune blistering disease of the skin and mucous membranes characterized by antibodies against the keratinocyte cell surface adhesion proteins desmoglein (Dsg) 1 and 3 (Stanley, 2003). There are two primary forms of pemphigus, pemphigus foliaceus (PF) and pemphigus vulgaris (PV). PF is characterized by autoantibodies against Dsg1, which cause blistering of the skin, but not mucous membranes, owing to loss of cell adhesion in the superficial epidermis. Mucosal PV is characterized by autoantibodies against Dsg3, which cause suprabasilar blistering of the mucous membranes. Mucocutaneous PV patients, who demonstrate both mucous membrane and skin involvement, usually develop additional autoantibodies against Dsg1. In both PV and PF, anti-Dsg antibodies are responsible for the positive direct and indirect immunofluorescence tests that are the pathognomonic feature of these diseases.

Experiments using passive transfer of autoantibodies to neonatal mice have demonstrated that the anti-Dsg antibodies in patients' sera are pathogenic (reviewed previously by Payne *et al.*, 2004). However, not all anti-Dsg antibodies cause disease. Epitope mapping studies have shown that the more pathogenic autoantibodies tend to bind the amino-terminal extracellular domain of Dsgs that is predicted to form the *trans*-adhesive interface between cells (Boggon *et al.*, 2002), whereas the less or non-pathogenic antibodies bind more membrane-proximal extracellular domains (Futei *et al.*, 2000; Sekiguchi *et al.*, 2001; Hacker-Foegen *et al.*, 2003; Li *et al.*, 2003).

Before the advent of corticosteroids, PV was a uniformly fatal disease due to severe blistering of the skin and oropharynx, with resulting malnutrition and sepsis. Currently, therapy for pemphigus relies on general immunosuppression, typically with corticosteroids, steroid-sparing agents, and/or adjunctive treatments such as intravenous immunoglobulin or plasmapheresis, most of which target the total antibody pool. Although mortality from pemphigus has decreased due to these therapies, a significant amount of patient morbidity and rarely mortality now results from side effects of these treatments. More specific antibody-targeted therapies for pemphigus would aim to suppress or eliminate only the anti-Dsg autoantibodies. The most specific therapy would be to target only the pathogenic autoantibodies. Antibodies could be targeted by their idiotype, an approach that has been previously reported for PF (Alvarado-Flores *et al.*, 2001). Alternatively, antibodies could be targeted based on their variable region gene usage, which has shown promise for

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Abbreviations: Dsg, desmoglein; ETA, exfoliative toxin A; HA, hemagglutinin; HRP, horseradish peroxidase; PF, pemphigus foliaceus; PV, pemphigus vulgaris; scFv, single-chain variable fragment; TBS, Tris-buffered saline;  $V_H$ , variable heavy-chain gene;  $V_H$ , variable region of the heavy chain;  $V_L$ , variable light-chain gene;  $V_L$ , variable region of the light chain

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other autoantibody-mediated diseases such as idiopathic thrombocytopenic purpura (Roark *et al.*, 2002). Either approach would require direct characterization of the individual (i.e., monoclonal) antibody populations that cause disease.

Prior studies using mouse-human heterohybridomas have isolated pathogenic and non-pathogenic mAbs from PV patients (Bhol and Ahmed, 2002; Qian *et al.*, 2006; Yeh *et al.*, 2006). However, heterologous cell fusion techniques are inefficient in capturing autoantibody repertoires, with cell lines often losing human chromosomes over time (Siegel, 2001). Anti-Dsg mAbs have also been generated using an active immune mouse model of PV, in which splenocytes from a Dsg3-deficient mouse were passively transferred to Rag2-deficient mice that express Dsg3, resulting in pathogenic and non-pathogenic anti-Dsg antibodies (Amagai *et al.*, 2000). Although some of these murine mAbs are pathogenic against human Dsg3, they are suboptimal for direct therapeutic targeting as they are mouse alloantibodies to murine Dsg3, with unclear clinical relevance to human anti-Dsg autoantibodies from PV patients.

We recently reported the cloning of pathogenic and non-pathogenic human PV mAbs by phage display (Payne *et al.*, 2005). One of the goals of cloning mAbs from pemphigus patients is to determine if therapy targeted to pathogenic antibodies is feasible. For such therapy to be practical, PV patients would have to demonstrate a limited genetic diversity of anti-Dsg pathogenic antibodies. Such genetic restriction was suggested by sequence analysis of the initial PV patient (PV(1)) library (Payne *et al.*, 2005). Reagents could then be developed to block pathogenic antibodies from binding Dsg target antigens and causing disease. Ultimately, these reagents would need to be effective among different patients. Here, we investigated both anti-idiotypic and  $V_H$  gene-targeted approaches to determine the feasibility of blocking pathogenic antibody populations in PV.

## RESULTS

### Genetic restriction of PV(1) mAb library

Previously, we reported the cloning of anti-Dsg mAbs from a patient with active mucocutaneous PV (Payne *et al.*, 2005). Genetic analysis of 63 sequences from the PV(1) mAb library demonstrated 43 unique mAbs based on V(D)J gene usage, heavy- and light- chain combinations, and somatic hypermutation. However, these 43 unique mAbs were encoded by a total of only seven variable heavy chain (VH) genes. When these antibodies were further subdivided by antigenic target and pathogenicity, only one to two VH genes were identified for each functional category (detailed in Table 1). Each of the 43 unique mAbs used one of 11 different D genes and one of two different joining heavy chain genes, but these gene usage patterns did not correlate with antigenic target or pathogenicity. Light-chain gene analysis also demonstrated genetic restriction, with nine different VL genes (Table 2) combining with three different joining light-chain genes. For anti-idiotypic reagent production, we chose representative pathogenic and non-pathogenic PV mAbs that were selected against Dsg3 alone (D3), or both Dsgs 3 and 1 (D31).

**Table 1.  $V_H$  gene restriction in the PV(1) phage display library**

Antigenic specificity	$V_H$ genes	Pathogenic	Non-pathogenic
Dsg 1	2	VH3-8	VH4-b
Dsg 3	3	VH3-07	VH3-30 VH1-e
Dsg 3+Dsg 1	2	VH1-4M28	VH4-04
Total	7	3	4

**Table 2.  $V_L$  gene restriction in the PV(1) phage display library**

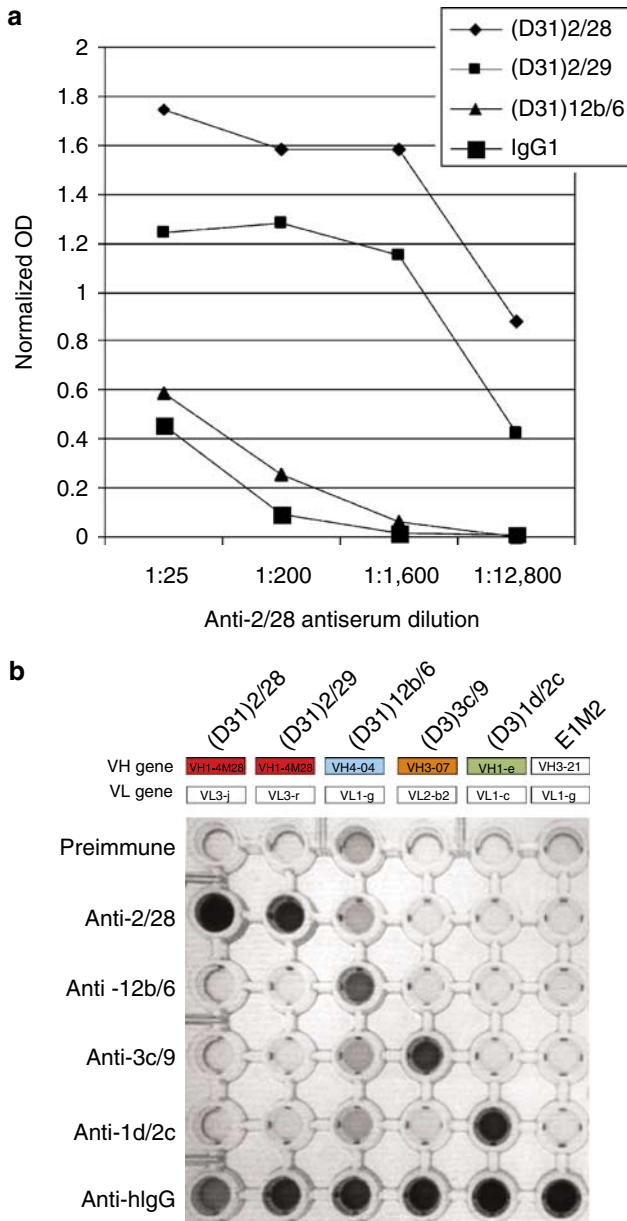
Antigenic specificity	$V_L$ genes	Pathogenic	Non-pathogenic
Dsg 1	2	VL2-a2	VL3h VKIII-L6
Dsg 3	4	VL2-b2	VL1-c VL1-g
Dsg 3+Dsg 1	3	VL3-r VL3-j	VL1-g
Total	9	4	5

ScFv nomenclature has been described previously (Payne *et al.*, 2005). Briefly, the single-chain variable fragment (scFv) designation includes the target antigens, (D3) or (D31), followed by a numeric designation for the heavy and light chain (i.e., 2/28), which is based on the V(D)J gene usage. Somatic mutation is indicated by small letter suffix; for example, (D3)3a/9 and (D3)3c/9 use the same heavy-chain ('3') and light chain ('9') variable region genes, but differ by somatic mutation in the heavy-chain variable region ('a' versus 'c').

### Production of anti-idiotypic antibodies against pathogenic and non-pathogenic PV mAbs

Recombinant pathogenic (D3)3c/9, (D31)2/28, and non-pathogenic (D3)1d/2c, (D31)12b/6 scFv PV mAbs were produced and purified by nickel chelation chromatography as described previously (Payne *et al.*, 2005). Rabbits were immunized with scFv preparations by a commercial vendor. The resulting rabbit antisera were first cleared against an irrelevant scFv and excess hemagglutinin (HA) peptide, followed by exhaustive clearing against polyclonal normal human IgG, in order to eliminate antibodies that reacted against non-idiotypic determinants.

ScFv antibody fragments express the monovalent antigen-binding sites of native immunoglobulin molecules. To evaluate the ability of rabbit antisera to bind PV mAbs in their native form, scFv were converted to bivalent full-length IgG1 molecules using the PIGG vector (Rader *et al.*, 2002).



**Figure 1. Anti-idiotype antisera specifically bind PV mAbs.** (a) Dose-dependent binding of PV mAbs by anti-idiotype antisera. Recombinant PV monoclonal IgG or monoclonal IgG1 lambda were adsorbed directly to ELISA plates and incubated with dilutions of anti-2/28 antiserum, followed by development with HRP-coupled anti-rabbit IgG. (b) Anti-idiotype antisera binding is specific. Recombinant PV monoclonal IgG or E1M2 negative control IgG (an anti-red blood cell antibody; Chang and Siegel, 1998) were adsorbed directly to ELISA plates and incubated with dilutions of preimmune sera or anti-idiotype antisera (1:1,000), followed by development with HRP-coupled anti-rabbit IgG. The bottom row was incubated only with HRP-coupled anti-human IgG as a control for IgG adsorption to the plate.

We detected dose-dependent binding of antisera to bivalent PV IgG by ELISA (Figure 1a). Figure 1a demonstrates that the antiserum raised against (D31)2/28 ('Anti-2/28') also bound (D31)2/29, an mAb which uses the same heavy chain (VH1-4M28) but has a different light chain. Anti-2/28 antiserum did

not bind to (D31)12b/6, which uses different heavy- and light- chain genes. To evaluate for potential crossreactivity of antisera with different PV IgG, PV IgG were directly adsorbed to ELISA plate wells and incubated with rabbit preimmune and immune sera. Figure 1b demonstrates that antisera specifically bound the immunizing mAb, with the exception of anti-2/28 antiserum, which also bound (D31)2/29. Antiserum raised against (D31)12b/6 did not crossreact with E1M2 IgG (an anti-red blood cell mAb (Chang and Siegel, 1998) produced using the same eukaryotic PIGG expression system), which uses the same VL gene (VL1-g). These data indicate that the epitopes recognized by some antisera are encoded by the heavy chain. Antisera did not recognize the denatured heavy or light chains of the target IgG by immunoblot (data not shown), suggesting that a conformational epitope was recognized.

#### Anti-Dsg idiotypes correlate with PV mAb V<sub>H</sub> gene usage

The phage display technique randomly pairs heavy- and light- chains during PCR construction of the scFv library, and thus mAbs isolated by phage display may not accurately reflect heavy and light chain pairings *in vivo*. If, however, the idiotypes of PV mAbs are displayed predominantly by the heavy chain, then development of anti-idiotypic reagents for pemphigus antibodies could be simplified.

To characterize the specificity of anti-idiotypic antisera, we tested each antiserum for its ability to inhibit binding of various PV mAbs to Dsg antigens by ELISA. In initial experiments, dose-response curves for anti-idiotypic antiserum inhibition were established, using preimmune serum as a control (Figure S1). Subsequently, ELISA inhibition assays were performed to evaluate the ability of each antiserum to inhibit binding of various PV mAbs to Dsg 3 and Dsg1. Figure 2 summarizes representative inhibition data for all PV mAb inhibition assays, using equivalent dilutions of anti-idiotypic antisera (1:20). Incubations in which the antisera were raised against an mAb using the same V<sub>H</sub> genes as the mAb tested by ELISA are highlighted in gray, demonstrating that antisera specifically inhibited Dsg binding by the original immunizing mAb, as well as mAbs that used the same V<sub>H</sub> but different V<sub>L</sub> genes. For example, the anti-2/28 antiserum inhibited (D31)2/28 as well as (D31)2/29, an antibody using the same V<sub>H</sub> but different V<sub>L</sub> genes.

Antisera did not inhibit mAbs that used different V<sub>H</sub> genes. Notably, the antiserum against (D31)2/28 pathogenic mAb did not inhibit binding of (D3)3c/9 pathogenic mAb to Dsg3, indicating that these two pathogenic antibodies do not share the same idiootype.

Additionally, the presence of somatic mutation did not appear to affect anti-idiotype antisera inhibition. For example, (D3)3c/9 and (D3)3a/9, as well as (D3)1d/2c and (D3)1g/2e, were similarly inhibited by anti-idiotypic antisera.

One non-pathogenic mAb, (D3)4/30, did not effectively immunize rabbits and was not substantially inhibited by any of the other four antisera, which were all produced against mAbs using different heavy-chain genes. PV(1) serum, obtained from the patient at the same time the phage display library was produced, showed 19% inhibition of total Dsg

VH gene	PV mAb	Anti-2/28	Anti-12b/6	Anti-3c/9	Anti-1d/2c	All 4
VH1-4M28	* (D31)2/28	84(5)/83(4)	-2	11/0.2	ND	
	* (D31)2/29	88(3)/89(7)	-2/1	1/5	ND	
VH4-04	* (D31)12b/6	3(2)/8(1)	92(4)/94	1(1)/12	-3	
VH3-07	* (D3)3c/9	2(3)	2	95(3)	5(1)	
	* (D3)3a/9	1(0)	ND	91(3)	2(3)	
	* (D3)3b/8	3(1)	ND	89(6)	-3	
VH1-e	* (D3)1b/3a	12	-6(23)	-4(23)	91(2)	
	* (D3)1d/2c	3	-2	0 (9)	94(3)	
	* (D3)1g/2e	1		5	81	
VH3-30	* (D3)4/30	7(1)	5(1)	4(0)	22(4)	
Polyclonal	PV(1) serum	4(1)	6(1)	11(2)	15(3)	19(2)

Figure 2. Antisera inhibition of PV antibody binding to Dsg3/Dsg1 by ELISA. Pathogenic mAbs are indicated by an asterisk. ND, not determined.

binding when all four antisera were used for inhibition in ELISA assays.

Taken together, these data suggest that the major idiotypes of the PV mAbs studied correlate with V<sub>H</sub> gene usage and are not significantly altered by somatic mutation or light chain gene usage, indicating that targeting of either idiotypes or V<sub>H</sub> genes may be a viable strategy for therapy.

**Anti-idiotypic antibodies block pathogenicity of PV mAbs**

We next investigated whether the *in vitro* inhibition of Dsg binding by ELISA translated into a functional block of antibody pathogenicity. We have previously shown that neonatal mouse injection is suboptimal for evaluation of pathogenicity of PV mAbs, due to differences in the specificity of mAbs for human versus mouse Dsg substrates (Payne *et al.*, 2005). Therefore, we evaluated the inhibition of mAb pathogenicity by a dispase assay using cultured primary human keratinocytes (Ishii *et al.*, 2005). Pathogenic PV mAbs (D3)3c/9, (D3)3a/9, (D31)2/28, and (D31)2/29 induced cell sheet dissociation in cultured human keratinocytes after preincubation with rabbit preimmune serum (top row, Figure 3). However, after preincubation with V<sub>H</sub>-specific antisera (anti-3c/9 antisera in the case of (D3)3c/9 and (D3)3a/9, and anti-2/28 antisera in the case of (D31)2/28 and (D31)2/29), the number of cell sheet fragments induced by PV mAbs was reduced (middle row, Figure 3). Inhibition of cell sheet fragmentation was not seen after preincubation of PV mAbs with antisera raised against PV mAbs using different V<sub>H</sub> genes (data not shown). Similar to our findings on ELISA, these data demonstrate that anti-idiotypic antisera specifically inhibit the pathogenicity of PV mAbs on the basis of their V<sub>H</sub> gene usage, despite the presence of different light chains or somatic mutation.

**Peptide phage display identifies sequences that bind PV mAbs according to V<sub>H</sub> gene usage**

Another method that might be used to target antibodies is to develop small peptides that bind pathogenic antibodies. To test the feasibility of this approach, we screened linear 12-mer and disulfide constrained 7-mer peptide phage display libraries with pathogenic (D31)2/29 PV mAb. Pools of phage-displayed peptides from the third round of selection demonstrated specific binding of both (D31)2/28 and (D31)2/29 mAbs, but not the nonpathogenic (D31)12b/6 mAb or an

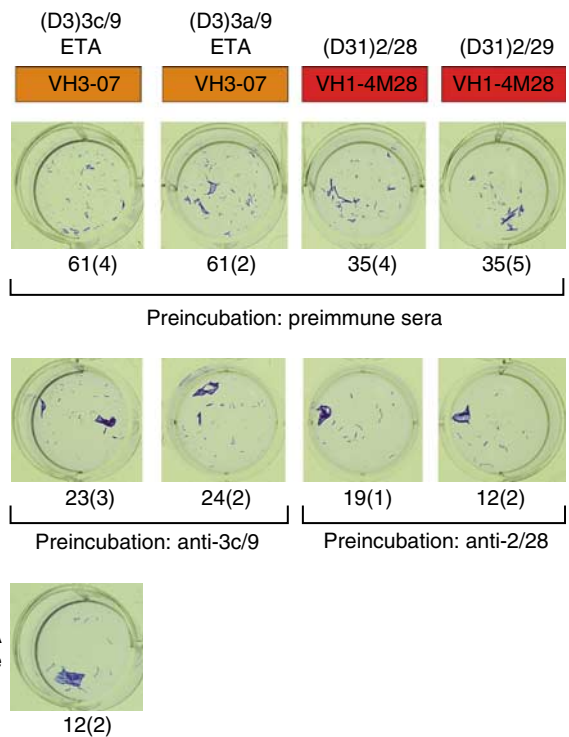


Figure 3. Anti-idiotypic antisera inhibit pathogenicity of PV mAbs according to V<sub>H</sub> gene usage. PV mAbs were preincubated with cleared rabbit preimmune sera or antisera before incubation with primary human epidermal keratinocytes. ETA was added to (D3) antibody incubations at a final concentration of 1 μg/ml in order to inactivate Dsg1. Intact cell sheets were released from the cell culture plate by treatment with dispase and subjected to mechanical shear stress. The ability of rabbit antisera to inhibit cell sheet dissociation by PV mAbs was quantified by counting the resulting number of cell sheet fragments, shown as a mean (SD).

IgG1 lambda mAb (Sigma, St Louis, MO) by ELISA (Figure S2), suggesting heavy-chain specificity of peptide binding. Preparations of individual binding phage clones were isolated and their displayed peptide amino-acid sequences were deduced by sequencing phage DNA (Figure 4). Interestingly, a consensus peptide sequence was identified. Neither the consensus sequence nor the individual peptide sequences aligned with any linear sequence in human desmosomal cadherins. Additionally, monoclonal peptide phage did not

Clone	Sequence
P4	ANKTSPFLMWRL
P5	WANKQPTIIWRS
P7	YSNKTPHLQWRL
P8	TDKTPELLWRVH
P9	AKNPPTLIWKHT
P10	QHNSKPNLYWRS
P14	TTLTLRH
Consensus	P- <sub>L</sub> -W <sub>R</sub> <sup>K</sup>

**Figure 4. Peptide sequences identified by peptide phage display screening using (D31)2/29 pathogenic PV mAb.** The consensus binding sequence is shown.

demonstrate direct binding to human Dsg3 by ELISA (data not shown), suggesting that these sequences do not mimic linear or conformational epitopes of Dsgs that may be involved in homo- or hetero-dimerization.

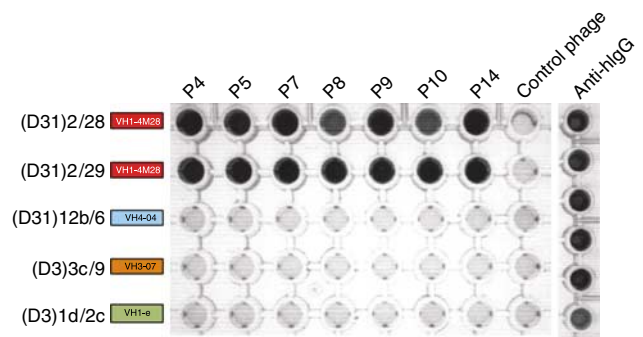
V<sub>H</sub>-specific binding of individual phage-displayed peptides to PV mAbs was confirmed by ELISA. PV mAbs were immobilized on microplate wells and incubated with peptide phage. Peptide phage demonstrated selective binding of (D31)2/28 and (D31)2/29 mAbs, but not other PV mAbs using different V<sub>H</sub> genes (Figure 5). Control peptide phage displaying a non-consensus sequence (DLNYFTLSSKRE), as well as wild-type peptide phage displaying pIII coat protein unligated to any additional peptide sequence (data not shown), did not show significant binding to any PV mAb.

Select peptide phage were tested for their ability to inhibit binding of PV mAbs to Dsg substrates by ELISA (Figure 6). Control phage displaying the non-consensus sequence did not demonstrate significant inhibition of PV mAb binding. P14 peptide phage also did not inhibit binding of any PV mAb. However, P4 and P7 peptide phage inhibited binding of (D31)2/29 by 78 and 83%, respectively, indicating that these peptides are anti-idiotypic reagents. Again, inhibition was dose dependent (data not shown). Although P4 and P7 peptide phage bound to (D31)2/28 mAb, they did not inhibit its binding to Dsg3.

Thus, as with the anti-idiotypic antibodies, specific binding of peptide phage to PV mAbs was mainly determined by V<sub>H</sub> gene usage. However, the binding of peptides to PV mAbs did not correlate with functional inhibition of mAb binding to Dsg3, indicating that the idiotype of PV mAbs may be modified by the light chain.

#### Anti-idiotypic antisera deplete pathogenic activity from PV(1) serum

Although the ELISA inhibition data (Figure 2) suggested that the pooled antisera did not inhibit the majority of total anti-Dsg binding in PV(1) serum, many anti-Dsg antibodies do not cause disease. We therefore tested whether the mAbs targeted by the antisera represented the critical pathogenic



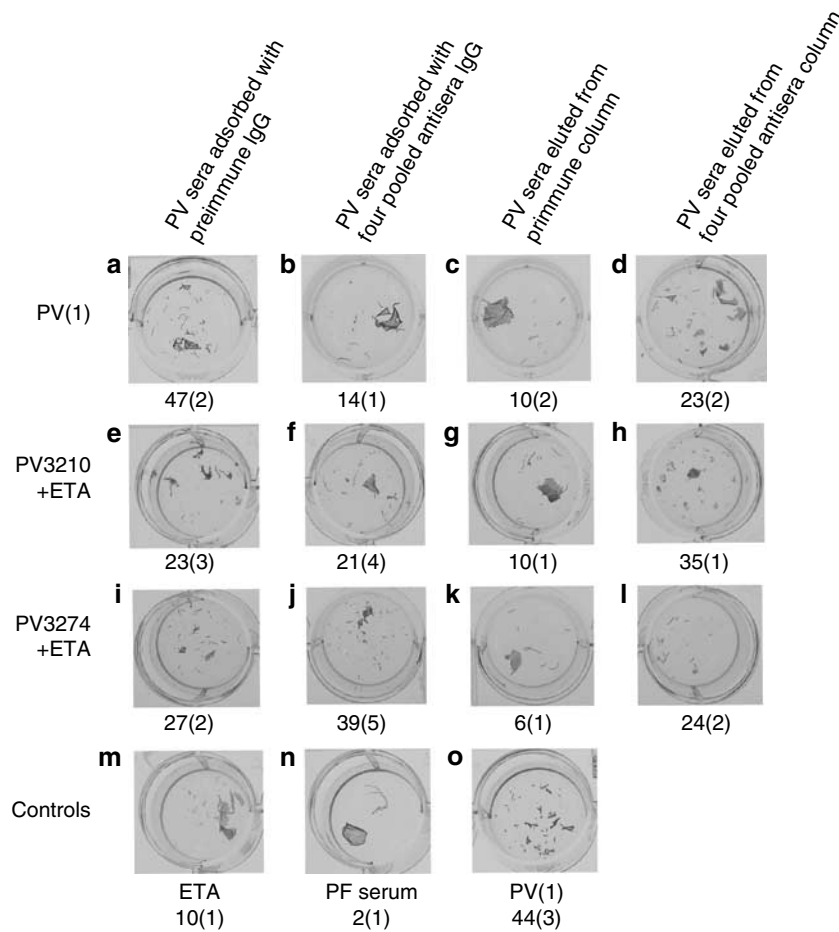
**Figure 5. Monoclonal peptide phage bind PV mAbs in a V<sub>H</sub> gene-specific manner.** PV mAbs were adsorbed to plates and incubated with 10<sup>9</sup> PFU of various phage-displayed peptides, followed by HRP-coupled anti-M13 secondary antibody. Control phage displayed a non-consensus peptide sequence. As a control for PV mAb adsorption to the plate, the last column was developed with HRP-coupled anti-human IgG.

VH gene	PV mAb	Control	P4	P7	P14
VH1-4M28	*(D31)2/28	2(6)	9(3)	12(7)	15(3)
	*(D31)2/29	15(3)	78(5)	83(9)	16(9)
VH4-04	(D31)12b/6	11(3)	10(14)	8(9)	13(2)
VH3-07	*(D3)3c/9	8(5)	7(6)	6(7)	5(2)
VH1-e	(D3)1d/2c	8(9)	14(13)	9(8)	8(21)

**Figure 6. Inhibition of PV mAb binding by monoclonal peptide phage clones.** Percent inhibition is shown as a mean (SD).

antibody populations using the keratinocyte dissociation assay. Collectively, the previous data demonstrate that both anti-idiotypic antibodies and small molecule reagents can specifically bind PV mAbs based on their heavy chain gene usage. Therefore, instead of targeting antibodies based on their idiotype, which may be a more restrictive functional definition and influenced by the light chain, we investigated a more general antibody targeting strategy based on antibody V<sub>H</sub> gene usage.

Rabbit preimmune and immune IgG were cleared only against an unrelated scFv (AM3-13, an anti-platelet antibody encoded by VH1-24 and VKI-O12/O2 genes) and excess HA peptide (and not against normal human IgG, which could clear antibodies that react against V<sub>H</sub> framework regions of the antibody). Antisera against non-pathogenic PV mAbs were included in case these antibodies contributed to pathophysiology when used in combination. Preimmune and immune IgG were immobilized on a solid phase matrix and used to determine whether pathogenic antibodies could be adsorbed from various PV sera. PV(1) serum is pathogenic in the dispase assay (Figure 7o). After adsorption with rabbit pre-immune IgG, PV(1) serum retained pathogenic activity against cultured human keratinocytes (Figure 7a). However, after adsorption with the four pooled PV antisera IgG, pathogenic activity of PV(1) serum was substantially reduced (Figure 7b). Acid elution of adsorbed proteins followed by



**Figure 7. Pooled antisera depletes pathogenic activity from PV(1) serum and binds pathogenic antibodies from different PV patients' sera.** Preimmune IgG and pooled antisera IgG were coupled to solid phase matrix and used to deplete various PV patients' sera, including PV(1), the patient from whom the PV(1) phage display library was produced. Owing to the low anti-Dsg1 titer of PV3210 and PV3274 sera, ETA was added to incubations using these sera. Both the depleted sera, as well as antibodies eluted from the rabbit preimmune and antisera columns, were evaluated by keratinocyte dissociation assay for pathogenic activity. The number of cell sheet fragments is shown as a mean (SD). See text for detailed discussion of (a-o).

neutralization demonstrated that pathogenic antibodies did not bind to the rabbit pre-immune column (Figure 7c). In contrast, PV(1) serum adsorbed by the four pooled PV antisera showed increased cell sheet fragmentation activity in the eluate, suggesting specific binding of pathogenic antibodies (Figure 7d). Two other PV patient sera, PV3210 and PV3274, did not show significant depletion of pathogenic activity by the four-pooled antisera (Figure 7, compare e-f, and i-j). However, pathogenic antibodies were eluted from both PV sera after adsorption with the four pooled antisera (Figure 7h and l), but not after adsorption with pre-immune IgG (Figure 7g and k). Exfoliative toxin A (ETA) (Figure 7m) or PF serum (Figure 7n), which only recognize Dsg1, do not demonstrate significant pathogenicity in the keratinocyte dissociation assay, which requires inactivation of both Dsg1 and Dsg3. These data suggest that the four  $V_H$  gene sets identified from the PV(1) patient library deplete a substantial proportion of the pathogenic activity from the patient's own serum. In addition, pathogenic antibodies using these  $V_H$  genes are shared among different PV patients,

although additional pathogenic autoantibody  $V_H$  genes are likely present in these other patients.

## DISCUSSION

### Autoantibody gene restriction in pemphigus

Genetic analysis of cloned antibodies from the PV(1) library demonstrated a restriction of autoantibody  $V_H$  gene usage, with different  $V_H$  gene usage by pathogenic and non-pathogenic antibodies (Table 1). Additionally, PV mAb  $V_H$  gene usage appeared to correlate with antibody function, with respect to both Dsg antigen binding and pathogenicity. Genetic restriction in the light chain repertoire was also observed (Table 2), suggesting functional importance (discussed below). The identification of limited genetic diversity in PV mAbs suggested that we may be able to improve the specificity and potentially the safety of pemphigus therapies by targeting only the anti-Dsg antibodies, as opposed to generally suppressing the immune system. The studies presented here aimed to explore the feasibility of such approaches.

Restricted patterns of heavy- and light- chain gene usage have been described for a number of autoimmune and naturally occurring immune responses, including idiopathic thrombocytopenic purpura, systemic lupus erythematosus, rheumatoid arthritis, cold agglutinins, HIV, and *Haemophilus influenzae* type b infection, among others (Adderson *et al.*, 1991; Silberstein *et al.*, 1991; Newkirk *et al.*, 1993; Ditzel *et al.*, 1996; Roben *et al.*, 1996; Roark *et al.*, 2002). The presence of antibody gene restriction in autoimmune and immune states is not surprising, as a limited number of antibody variable region genes would be expected to confer the ability to bind a specified target antigen. For example, antibodies that bind the human red cell Rh(D) antigen use  $V_H$  gene segments that encode polypeptides with higher isoelectric points, which has been suggested to facilitate binding of epitopes in the negatively charged red cell membrane (Boucher *et al.*, 1997). In the case of PV and PF, Dsg antigens are largely conserved among patients, with the possible exception of case reports demonstrating Dsg3 and Dsg1 haplotypes, respectively, that are associated with disease (Martel *et al.*, 2001; Capon *et al.*, 2006). Owing to the conservation of Dsg antigens among patients, it is logical that autoantibody  $V_H$  gene usage may be restricted and shared among patients and thus may serve as a therapeutic target.

It is likely that the phage display technique does not capture all of a patient's anti-Dsg mAbs, owing to potential bias that may be introduced by the selection process and how well individual phage clones grow in culture. This was supported by our ELISA inhibition assays (Figure 2), which demonstrated that all four antisera inhibited only 19% of total PV(1) serum Dsg3 binding. A similar percent inhibition of PV(1) binding was observed when all four antisera were used to deplete PV(1) sera before the Dsg3 ELISA binding assay (data not shown), indicating that the percent inhibition observed in competitive binding assays was not secondary to relative affinities of PV serum IgG for Dsg versus antisera substrates. One non-pathogenic mAb, (D3)4/30, was not specifically targeted by the antisera and presumably contributed to residual Dsg3 binding by the depleted serum. Of note, the 19% inhibition observed with all four antisera was lower than the total inhibition expected when each of the four antisera was used individually to inhibit PV(1) binding (cumulative total inhibition approximately 36%). This finding may be explained by cooperative binding of PV autoantibodies to Dsg (Tsunoda *et al.*, 2006; ASP, unpublished data).

Although the mAbs cloned from the PV(1) patient were likely not inclusive of all serum anti-Dsg antibodies, our data suggest that we have identified critical pathogenic antibody populations, as targeted  $V_H$  gene depletion of PV(1) serum with the four antisera resulted in a marked reduction in PV(1) serum pathogenicity (Figure 7). These findings argue against the possibility that the genetic restriction observed was solely owing to inefficient isolation of disease-relevant antibody clones from the patient library. These data suggest that for an individual patient, we have identified restricted populations of anti-Dsg mAbs by phage display, generated  $V_H$  gene-targeted reagents that specifically bind and inhibit these anti-

Dsg mAb populations, and used these reagents to deplete a majority of PV(1) serum pathogenic activity.

#### Shared $V_H$ gene usage among pemphigus patients

The feasibility of more generally applicable  $V_H$  gene-targeted therapies for PV relies on the presence of shared pathogenic antibodies among patients. The paradigm of  $V_H$  gene restriction in autoimmune diseases suggests that a limited number of  $V_H$  genes may be able to cause disease. Our data demonstrate that antibody function and the binding and inhibition of anti-idiotypic reagents correlate with  $V_H$  gene usage (Table 1 and Figures 1 and 2). Thus, instead of screening PV patients for shared antibody idiotypes, which may be more difficult to detect owing to their presumed low level of representation in the total autoantibody repertoire, we screened two independent PV patients for pathogenic antibodies that may use the same  $V_H$  genes. Because recent experiments in mice have suggested that combinations of non-pathogenic PV mAbs may result in pathogenic activity (Kawasaki *et al.*, 2006), we used all four antisera for antibody adsorption.

The four antisera against  $V_H$  genes identified from the PV(1) patient adsorbed pathogenic antibodies from two different PV patients (Figure 7h and i), suggesting that some or all of these  $V_H$  genes are used by pathogenic antibodies in these two patients. However, the inability to deplete significant pathogenic activity from the two patient sera (Figure 7f and j) suggests either a significantly higher titer of antibodies using the  $V_H$  genes in these sera, or more likely, additional  $V_H$  gene usage by pathogenic antibodies in these two patients. We have demonstrated that the antisera are specific for certain  $V_H$  genes (Figures 1 and 2), thus making the possibility of crossreactive binding of antisera to unrelated PV mAbs unlikely. Ultimately,  $V_H$  gene analysis of more PV patient autoimmune repertoires may identify additional disease-associated  $V_H$  genes for targeting.

#### Anti-idiotypic antibodies bind and inhibit PV mAbs according to $V_H$ gene usage

With the phage display technique, heavy and light chains are randomly paired during PCR construction. However, light-chain shuffling experiments have demonstrated that reproducible heavy- and light- chain combinations are isolated after (but not before) phage library selection (Roben *et al.*, 1996; Roark *et al.*, 2002). These findings indicate that a preferential association of heavy and light chains is detected when the functional constraint of antigen binding is imposed. Thus, the heavy- and light- chain combinations in mAbs isolated by phage display may in fact represent biologically relevant pairings.

Crystal structures of antibody variable regions have shown that the antigen binding pocket is formed at the interface of the  $V_H$  and  $V_L$  genes (Rose *et al.*, 1993; Ay *et al.*, 2000). These and other reports have demonstrated that the  $V_H$  gene encodes the majority of the antigenic binding determinants, primarily through the CDR3 (Arevalo *et al.*, 1993; Ban *et al.*, 1995; Graille *et al.*, 2000). Other studies have shown that the CDR3 of the heavy chain is sufficient for antigen recognition

(Barbas *et al.*, 1995), even in the absence of the light chain (Bourgeois *et al.*, 1998; Zwick *et al.*, 2004). The presence of different light chains has been proposed to influence antibody affinity in addition to epitope specificity (Ibrahim *et al.*, 1995; Ohlin *et al.*, 1996; Hoet *et al.*, 1999). Consistent with this, our peptide phage inhibition data indicate the relevance of the light chain for some antibody idiotypes, as P4 and P7 peptide phage bound both (D31)2/28 and (D31)2/29 pathogenic antibodies but only inhibited (D31)2/29 from binding to Dsg3.

Despite fine differences in anti-idiotypic inhibition of PV mAbs by monoclonal peptide phage, our data demonstrate that the binding of anti-idiotypic antibodies and peptide reagents to PV mAbs correlates with  $V_H$  gene usage (Figures 1, 2, and 5). Neither different light chains nor somatic mutation of the variable region outside the CDR3 affected the binding or inhibition of anti-idiotypic antisera (Figures 1–3). These data suggest that PV mAb  $V_H$  genes identified by phage display represent feasible targets for therapy.

#### Identification of a peptide consensus sequence for PV mAb binding

Using peptide phage display, we have identified linear peptide sequences that specifically bind pathogenic PV mAbs (Figures 4 and 5). These reagents, similar to the anti-idiotypic antisera, are  $V_H$ -specific in their binding of PV mAbs. Interestingly, the consensus peptide sequence includes a tryptophan residue (Figure 4). Membrane distal tryptophan residues form a critical part of the *trans*-adhesive interface between cells for the classical cadherins, whose extracellular domain structures have been determined (Shapiro *et al.*, 1995; Nagar *et al.*, 1996; Boggon *et al.*, 2002). However, the consensus sequence identified does not correlate with any linear sequence in human Dsgs 1–4 or human desmocollins 1–3. Additionally, peptide phage displaying the consensus sequence did not demonstrate specific binding directly to Dsg3 by ELISA, and injection of peptides into normal human skin did not result in any tissue pathology (data not shown). These data suggest that the peptide sequences identified do not mimic the desmosomal cadherin adhesive interface but instead encode sequences for direct binding of specific  $V_H$  genes. Such reagents could be chemically modified to optimize pathogenic antibody adsorption from PV sera (discussed below).

#### Therapeutic implications

Recently, rituximab, a monoclonal antibody against the B-cell surface antigen CD20, has shown promise for the treatment of autoimmune diseases including pemphigus (España *et al.*, 2004; Arin *et al.*, 2005; Ahmed *et al.*, 2006). The exact mechanism of action is unclear, as less than 20% of circulating plasma cells express CD20, but rituximab is thought to deplete B-cell precursors that may ultimately give rise to autoantibody-secreting plasma cells. Nevertheless, the anecdotal success of rituximab underscores the importance of the humoral immune response for perpetuating disease. As an alternative to broadly targeting antibody-producing cells, treatments could be aimed directly

against the disease-causing anti-Dsg antibodies or the B cells that produce them.

The cloning of human PV mAbs may identify novel strategies for such direct antibody targeting. Our current data suggest that treatments could be designed on the basis of autoantibody  $V_H$  gene usage. In addition, the identification of peptides that specifically bind PV mAbs (Figure 5) provides proof of principle that small molecule reagents can also be identified that can discriminate among PV mAbs based on their  $V_H$  gene usage. These small molecule reagents are more practical than rabbit anti-idiotypic antibodies for the development of therapeutic intervention strategies, although the latter approach is also amenable to humanization strategies through phage display (Steinberger *et al.*, 2000). Such antibody-targeted reagents could be coupled to columns as an adjunct for plasmapheresis to improve the efficiency of pathogenic antibody removal from patients' sera. Alternatively, antibody-specific reagents could be linked to B cell superantigens as a method of  $V_H$ -targeted B-cell deletion, similar to what has previously been described for the selective deletion of VH3 family genes by *Staphylococcus aureus* protein A (Goodyear and Silverman, 2003, 2004). Although beyond the scope of the current study, these novel approaches to antibody-targeted therapy offer hope for potentially safer and more effective treatments for pemphigus. In addition, the  $V_H$  gene-targeting approach described may have implications for the treatment of other genetically restricted antibody-mediated diseases.

#### MATERIALS AND METHODS

All experiments were conducted in accordance with the Declaration of Helsinki Principles for Human Tissue Research and were approved by the Necessary Regulatory Boards of the University of Pennsylvania.

#### Production of anti-idiotypic antisera

ScFv mAbs were produced in the Top10F' strain of *E. coli* (Invitrogen, Carlsbad, CA) and purified by nickel chelation affinity chromatography as described previously (Payne *et al.*, 2005). Rabbit antisera were commercially produced by Cocalico Biologicals Inc., according to the vendor's standard protein immunization protocol.

Rabbit antisera were first cleared against an irrelevant human scFv, AM3-13 (encoded by VH1-24 and VKI-O12/O2 genes), along with excess HA peptide. To produce the column, 520  $\mu$ g of AM3-13 and 60  $\mu$ g of HA peptide (Sigma, St Louis, MO) were coupled to 500  $\mu$ l of Affigel 15 matrix (BioRad, Hercules, CA) according to the manufacturer's instructions. Rabbit antiserum (500  $\mu$ l) was incubated with the coupled beads by end-over-end rotation overnight at 4°C. The flow through the column was collected and combined with the flow through after washing with one bed volume of PBS, pH 7.4. Clearing was evaluated by testing the antisera for depletion of anti-HA activity by ELISA.

To produce an affinity column comprising normal human IgG, 10 mg of human IgG (Sigma) was coupled to 1 ml of Affigel 10 matrix (BioRad) according to the manufacturer's instructions. 1 ml of rabbit antiserum was incubated with the human IgG affinity column by end-over-end rotation for 8–16 hours at 4°C and the flow through

from the column was collected and combined with the flow through after washing with one bed volume of PBS, pH 7.4. The column was then washed with an additional 10 bed volumes of PBS and bound IgG was eluted with six bed volumes of 100 mM glycine, (pH 2.5). The elution fractions were neutralized with 1/10 volume of 1 M Tris, (pH 7.5), and the column was neutralized by washing with 20 bed volumes of PBS, (pH 7.4). Binding and elution steps were repeated for 3–8 times, until the A280 of the eluate from the column stabilized. The flow through fractions (comprising the cleared antisera) were pooled and concentrated by Centricon YM-10 ultrafiltration (Millipore, Billerica, MA).

To produce rabbit anti-idiotypic columns, rabbit IgG was purified from preimmune and immune sera by protein A adsorption and elution with acid glycine. The eluate was dialyzed against PBS, (pH 7.4). Purified rabbit preimmune or antisera IgG (2.5 mg) was coupled to 500  $\mu$ l of Affigel 10 matrix (BioRad) as above. PV sera (250  $\mu$ l) was adsorbed with the rabbit IgG affinity column overnight at 4°C and the flow through and eluate were collected as described above.

### Production of bivalent IgG1 from monovalent scFv mAbs

The eukaryotic PIGG expression vector was provided by Carlos Barbas (Scripps Research Institute). Primers used to subclone scFv mAbs into the PIGG vector appear in Table S1. PV mAbs, contained in the scFv phagemid expression vector pComb3X, were used as the PCR template for the antibody variable region amplifications. E1M2 Fab in the phagemid expression vector pComb3H (Roark *et al.*, 2002) was used as the PCR template for the constant region of the lambda light chain ( $C_L$ ).

The variable region of the heavy chain ( $V_H$ ) was amplified with a 5' primer based on the sequence of PIGG-A, which introduces an *SacI* cloning site (Rader *et al.*, 2002), along with 3' primer HSCG1234B (Barbas *et al.*, 2001), which includes the endogenous *Apal* restriction sequence at the start of the IgG1 CH1 region. The PCR reaction was purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA) and digested with *SacI* and *Apal* (New England BioLabs, Ipswich, MA). The *SacI*–*Apal* fragment was purified by agarose gel electrophoresis using the Qiaquick Gel Extraction Kit (Qiagen) and was subcloned into the *SacI*–*Apal* site in the PIGG vector.

The variable region of the light chain ( $V_L$ ) was amplified with a 5' primer based on the sequence of PIGG-C (Rader *et al.*, 2002), which introduces a *HindIII* cloning site and abolishes the internal *SacI* cloning site, which would otherwise have been amplified from the pComb3X vector. The 3' primer for the  $V_L$  region hybridizes in reverse orientation to the 3' end of the  $V_L$  sequence. The  $C_L$  region was amplified from E1M2 Fab using 5' primer HLC-F (Barbas *et al.*, 2001) and 3' Lead B reverse primer (Barbas *et al.*, 2001). The  $V_L$  and  $C_L$  PCR fragments were purified by agarose gel electrophoresis as above and quantitated by relative ethidium bromide fluorescence. The  $V_L$ – $C_L$  region was produced by overlap PCR, using approximately 50 ng each of the  $V_L$  and  $C_L$  PCR fragments as the template, and the 5' light chain and 3' lead B reverse primers. The  $V_L$ – $C_L$  overlap PCR reaction was PCR purified, digested with *HindIII* and *XbaI* (New England BioLabs), and purified by agarose gel electrophoresis before subcloning in the *HindIII*–*XbaI* site of the PIGG vector. All recombinant constructs were verified by automated sequencing.

### PIGG vector expression

Endo-free plasmid maxipreps (Qiagen) of recombinant PIGG vectors were prepared for eukaryotic cell transfection into 293T cells. PIGG vector transfection was performed using jetPEI reagent (ISC Bio-Express, Kaysville, UT) according to the standard protocol, using five 10 cm cell culture plates at approximately 90% cell density and DMEM plus 10% ultralow IgG fetal bovine serum (Gibco/Invitrogen, Carlsbad, CA) as the harvest media. Expressed antibody was harvested from the cell culture supernatant at 3 days and again at 6 days. Non-adherent cells were removed from the supernatant by centrifugation, and the media was neutralized by the addition of 1/100 volume 1 M Tris pH 7.5. IgG was purified from the culture supernatant by rotation with protein A agarose beads (Invitrogen) for 2 hours at room temperature. The beads were transferred to a disposable chromatography column (BioRad), washed with 20 column volumes of PBS, and eluted with 6 column volumes of 100 mM glycine (pH 3). Eluted fractions were neutralized with 1/10 volume 1 M Tris pH 7.5, and the approximate IgG concentration was estimated by absorbance at 280 nm using an extinction coefficient of 1.43. Samples were dialyzed into PBS (pH 7.4) and concentrated to approximately 1  $\mu$ g/ $\mu$ l using Centricon YM-3 spin columns (Millipore, Billerica, MA). Antibody concentration was confirmed by non-reducing SDS-PAGE followed by Coomassie staining, using a known amount of monoclonal lambda IgG1 (Sigma) as a concentration standard for reference. Antibody binding to Dsg was confirmed by ELISA (Rhigene/MBL, Woburn, MA) according to the manufacturer's protocols.

### Peptide phage display and techniques

PhD-12 and PhD-C7C peptide phage display libraries (New England BioLabs) were screened with PV IgG according to the manufacturer's instructions, alternating between protein A and protein G magnetic beads (New England BioLabs) for antibody capture. Phage clones were isolated from round 3 of screening for sequencing according to the manufacturer's protocols. Unique clones were subsequently characterized by ELISA binding and inhibition assays.

### ELISA binding and inhibition assays

For anti-idiotypic antiserum binding assays, PV monoclonal IgG were adsorbed directly to ELISA plates at a concentration of 10  $\mu$ g/ml in PBS overnight at 4°C. The antigen was discarded, and the plate was blocked for 1 hour at 37°C in blocking buffer (0.1 M NaHCO<sub>3</sub>, pH 8.6, 5 mg/ml BSA, 0.02% NaN<sub>3</sub>). After blocking, the plate was washed with Tris-buffered saline (TBS) containing 0.1% Tween-20. For antisera evaluation, cleared rabbit pre-immune and immune sera were incubated at a dilution of 1:1000 (or varying dilutions) on each well and detected with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibodies (Dako, Carpinteria, CA), which demonstrate minimal crossreactivity with human IgG. As a control for mAb adsorption to microplate wells, HRP-conjugated anti-human IgG (Research Diagnostics Inc., Concord, MA) was also reacted with wells. ABTS (2,2'-Azino-di[3-ethylbenzthiazoline-sulfonate], Roche Applied Science, Indianapolis, IN) was used as the substrate for ELISA reactions.

For peptide phage binding assays, 0.5  $\mu$ g of rabbit anti-human Fc was adsorbed to ELISA plates overnight at 4°C in 0.1 M NaHCO<sub>3</sub> (pH 9.6). The antigen was discarded, and the plate was blocked as above. Wells were subsequently incubated with 0.5  $\mu$ g of various PV

mAbs in TBS-0.5% Tween-20 for 2 hours at 37°C. After washing with TBS-0.5% Tween-20, 10<sup>9</sup> PFU of monoclonal peptide phage in TBS-0.5% Tween-20 were incubated in each well for 1 hour at room temperature, followed by HRP-conjugated anti-M13 secondary antibody (Amersham Biosciences/GE Healthcare, Piscataway, NJ) in blocking buffer and development with ABTS substrate. As a control to evaluate PV mAb adsorption to microplate wells, 50 ng of PV mAbs was added to the rabbit anti-human Fc-coated wells, and then detected with HRP-conjugated anti-human IgG.

For ELISA inhibition assays, anti-idiotypic reagents were pre-incubated with PV mAbs for 1 hour at 37°C before incubation on Dsg3 or Dsg1 ELISA plates (Rhigene/MBL, Woburn, MA), which were otherwise processed according to the manufacturer's instructions. Anti-idiotypic antisera were effective and dose responsive at dilutions ranging from 1:3 to 1:6,400, whereas peptide phage were used in inhibition assays from 10<sup>8</sup> to 10<sup>10</sup> PFU/well. PV scFv binding, detected with HRP-conjugated anti-HA secondary antibody (Roche Applied Science), was titrated to a final OD<sub>450</sub> reading of 0.4–1.0, which was within the linear range of detection.

### Keratinocyte dissociation assay

The keratinocyte dissociation assay was performed as previously described (Payne *et al.*, 2005), using either PV scFv at 5 µg/ml or PV sera at dilutions of 1:5–1:7.5 in 500 µl total volume defined keratinocyte-serum free media (Gibco/Invitrogen). For antibodies that only recognize Dsg3, 1 µg/ml ETA was added to wells for the last 2 hours. For antisera depletion assays, 200 µl of PV sera flow through (representing a volume of 100 µl sera, combined with the first 1-volume fraction of the PBS wash) was incubated with cells in a total volume of 500 µl, with or without ETA treatment. Dissociation assays used the total eluate from the antisera columns, concentrated to a volume of 100 µl (representing a starting volume of 250 µl sera).

### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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### SUPPLEMENTARY MATERIAL

**Table S1.** Primers used for PV mAb PIGG vector construction.

**Figure S1.** Antisera inhibition of PV mAb binding to Dsg3 is dose dependent.

**Figure S2.** Peptide phage pools selectively bind pathogenic PV mAbs.

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