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Defining Mechanisms of Autoimmunity in Pemphigus Vulgaris

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Abstract

A prominent question in the field of autoimmunity is how these diseases arise. Currently, the etiologies of many autoimmune diseases remain unclear. The work described here provides insight into this question in the context of pemphigus vulgaris (PV), a prototypic autoimmune disease characterized by serum autoantibodies to desmoglein (Dsg) 3. We utilize a combination of both antibody phage display and heterohybridoma to probe the anti-Dsg3 antibody repertoires of patients with PV. We first address whether a cohort of four patients with active disease demonstrate any shared characteristics in their anti-Dsg3 antibody repertoires (Chapter 2), and if so, why. We find shared utilization of VH1-46 in at least anti-Dsg3 antibody across all four patients, and that these VH1-46 autoantibodies require few to zero somatic mutations to bind Dsg3, which may explain their presence in all four patients studied. Based on this, we propose a “shared VH gene usage” theory in the development of PV and investigate rotavirus as a potential viral trigger of this autoimmune disease, as VH1-46 has also been observed in the antibody response to the rotavirus intermediate capsid protein VP6 (Chapter 3). We determine that, while uncommon, it is possible for a VH1-46 antibody to cross-react to both Dsg3 and VP6, and this can occur through either V(D)J recombination or somatic hypermutation. In addition, a subset of these cross-reactive antibodies can both inhibit rotavirus replication and Dsg3 adhesion in vitro, indicating that these cross-reactive antibodies may have a role in the context of both rotavirus infection and PV. Our findings indicate that VH1-46 may persist in the anti-Dsg3 antibody repertoires of certain people due to the ability of some of these antibodies to cross-react to a foreign antigen and thus provide protection during infection. Ultimately, the data presented here provide a better understanding of the pathophysiology of this disease as well as potential etiologies of Dsg3 antibody reactivity.

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PEER REVIEWED PUBLICATIONS

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ATTRIBUTIONS

- Chapter 2 contains data from publication 3 listed on the previous page.
- Chapters 2 and 3 contain data from publication 1 listed on the previous page.
- Figure 1-4 is taken from Chan,T.D. *et al.* Elimination of germinal-center-derived self-reactive B cells is governed by the location and concentration of self-antigen. *Immunity.* 37, 893-904 (2012).
- Figure 1-5 A is adapted from Ishii,K. Identification of desmoglein as a cadherin and analysis of desmoglein domain structure. J. Invest Dermatol. 127, E6-E7 (2007).
- Figure 1-5 B is taken from Boggon,T.J. et al. C-cadherin ectodomain structure and implications for cell adhesion mechanisms. Science 296, 1308-1313 (2002).
- Figure 1-6 is adapted from Wolff K, Goldsmith LA, Katz SI, Gilchrest BA, Paller AS, Leffell DJ: *Fitzpatrick's Dermatology in General Medicine,* 7th Edition.
- Figure 1-8 is a summary of data from:

  Weitkamp,J.H. *et al.* Infant and adult human B cell responses to rotavirus share common immunodominant variable gene repertoires. *J Immunol.* 171, 4680-4688 (2003).

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ABSTRACT

DEFINING MECHANISMS OF AUTOIMMUNITY IN PEMPHIGUS VULGARIS

Michael Jeffrey Tejada Cho

Aimee Payne

A prominent question in the field of autoimmunity is how these diseases arise. Currently, the etiologies of many autoimmune diseases remain unclear. The work described here provides insight into this question in the context of pemphigus vulgaris (PV), a prototypic autoimmune disease characterized by serum autoantibodies to desmoglein (Dsg) 3. We utilize a combination of both antibody phage display and heterohybridoma to probe the anti-Dsg3 antibody repertoires of patients with PV. We first address whether a cohort of four patients with active disease demonstrate any shared characteristics in their anti-Dsg3 antibody repertoires (Chapter 2), and if so, why. We find shared utilization of VH1-46 in at least anti-Dsg3 antibody across all four patients, and that these VH1-46 autoantibodies require few to zero somatic mutations to bind Dsg3, which may explain their presence in all four patients studied. Based on this, we propose a “shared VH gene usage” theory in the development of PV and investigate rotavirus as a potential viral trigger of this autoimmune disease, as VH1-46 has also been observed in the antibody response to the rotavirus intermediate capsid protein VP6 (Chapter 3). We determine that, while uncommon, it is possible for a VH1-46 antibody to cross-react to both Dsg3 and VP6, and this can occur through either V(D)J recombination or somatic hypermutation. In addition, a subset of these cross-reactive antibodies can both inhibit rotavirus replication and Dsg3 adhesion *in vitro*, indicating that these cross-reactive antibodies may have a role in the context of both rotavirus infection and PV. Our findings

indicate that VH1-46 may persist in the anti-Dsg3 antibody repertoires of certain people due to the ability of some of these antibodies to cross-react to a foreign antigen and thus provide protection during infection. Ultimately, the data presented here provide a better understanding of the pathophysiology of this disease as well as potential etiologies of Dsg3 antibody reactivity.

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CHAPTER 1: INTRODUCTION

Canonically, one typically thinks of an immunologist as one who studies the immune response to a foreign insult, such as a virus or bacterium. I am particularly interested in the other side of that coin; instead of trying to understand how the body reacts to a foreign antigen, I am interested in how the body prevents itself from reacting to self-antigens. The focus of my thesis has been to define the developmental events that lead to an autoimmune response, which if better understood, would allow investigators to design better treatment strategies for the 24 million people in the United States that currently suffer from an autoimmune disease[1].

1.1 Overview of B cell Development

1.1.1 B cell Receptor Development

The adaptive immune system provides the humoral immunity needed to protect an organism from past infections by conferring immunological memory to the specific insult. Antigen-experienced B and T cells can interact with various foreign insults in an antigen-specific manner to clear secondary infections at a much faster rate than primary infections. This antigen specificity in both B and T cells arises from the B cell receptors (BCRs) and T cell receptors (TCRs). Both of these antigen-binding surface molecules are generated by a process called V(D)J recombination, which will now be described from the perspective of a B cell.

Common lymphoid progenitors give rise to pro-B cells via the induction of E2A and early B cell factor (EBF), which are two transcription factors unique to the B cell

lineage[2, 3]. Pro-B cells express a pro-BCR and have yet to initiate DNA rearrangements at the immunoglobulin (Ig) heavy chain loci[4] that will ultimately generate the heavy chain of the BCR. E2A and EBF induce the expression of recombinase-activating genes (RAG) 1 and 2 alongside other B cell-related proteins such as paired box transcription factor 5 (Pax5)[5, 6], which upregulates expression of CD19, a B cell co-receptor for the BCR[7]. Expression of RAG1/2 allows the pro-B cell to undergo VDJ recombination[8, 9], which, in an error-prone manner, will bring together a DH and JH segment together via a DH-JH join. Subsequently, a V segment will also be attached to the DH-JH join, forming a complete heavy chain **(Figure 1-1)**, which is then tested for the ability to signal downstream when paired with the surrogate light chain proteins lambda 5 and VpreB; termed the pre-BCR[10]. If this pre-BCR is unable to transduce signals downstream through the other components of the BCR complex such as Igα and Igβ[10, 11], as is the case for two thirds of the rearrangements that are out of frame, further heavy chain rearrangements can occur at the rearranged locus, and if that locus has exhausted all VH segments, heavy chain rearrangements can also occur on the second chromosome. It has been shown that ~45% of pro-B cells are lost at this stage of development. Allelic exclusion is also enforced at this stage to prevent the expression of two distinct heavy chains on the surface of a single B cell[12, 13], and occurs post-pre-BCR signaling by limiting the number of functional heavy chains to one per B cell[14], likely through both regulated and unregulated mechanisms.

With the expression of a functional heavy chain, the cell becomes a pre-B cell, and VJ rearrangement then occurs at the light chain loci, where VL and JL segments are rearranged to form a light chain; the final portion of the complete BCR. Non-productive rearrangements at the light locus are mitigated in a similar fashion as the heavy locus[15-

[18]. This newly formed, productive light chain can then pair with the functional heavy chain, and again in association with Ig-α and Ig-β, is further subjected to test its functionality in inducing downstream signaling after BCR engagement. Allelic exclusion, as observed at the heavy chain locus, is also enforced at this stage of BCR development. In addition, isotypic exclusion of the light chain prevents the expression of both a κ and a λ light chain simultaneously[14, 19]. There does exist a small population of B cells in both mouse and man that demonstrate allelic inclusion; that is, surface expression of both a κ and a λ light chain[20-22], but this is beyond the scope of this thesis.

V(D)J recombination can utilize any one of the functional 38-46 VH segments, 23 DH segments, and 6 JH segments to form a heavy chain. At the light chain locus, there are 29-33 VL segments, 4-5 JL segments, 31-36 VK segments, and 5 JK segments[23-27]. The large number of gene segments, paired with the error-prone polymerase η and the addition of non-templated nucleotides via terminal deoxynucleotidyl transferase, gives this stochastic process of DNA rearrangement the ability to generate the diversity observed in the human adaptive immune system, where there are upwards of 10^{11} number of antigen specificities within a single individual[28, 29]. This large diversity is primarily due to the random pairing of heavy and light chains, and the hypervariable complementarity-determining region (CDR) 3, which spans the VDJ junction on the heavy chain **(Figure 1-2)**. CDRs are responsible for the main contact between the BCR and its cognate antigen, and are surrounded by framework regions (FWRs), that are primarily responsible for structure and stability of the BCR. The CDR1 and CDR2 on the heavy chain are encoded entirely by the VH gene segment. Interestingly, it has been described that the heavy chain CDR3 the most important in determining antibody:antigen specificity; transgenic mice expressing a single VH gene segment

coupled with the full diversity of DH and JH gene segments were fully capable of generating high affinity clones for several different types of antigens[30].

However, the process of V(D)J recombination, which can provide the varied range of antigen specificity needed over the course of a lifetime, is a double-edged sword, as it is certain to confer reactivity to self. Therefore, the immune system has several tolerance checkpoints in place to prevent these self-reactive clones from developing into cells that could cause autoimmunity. It is at the immature B cell stage when the nascent BCR is first tested for autoreactivity to antigens present in the surrounding environment of the bone marrow niche[31].

It has been shown previously that upon high affinity interactions with self-antigen in the bone marrow, B cells undergo several mechanisms of central tolerance to prevent autoimmunity. This first of these mechanisms is receptor editing, which occurs after functional rearrangements at either the light and heavy chain loci. Given that a single B cell clone has multiple light chain loci in its genome, a self-reactive pre-B cell clone can arrest at this stage and reactivate RAG 1 and 2 to undergo further rearrangements at the light locus in an attempt to abolish self-reactivity[32]. This process first occurs at the kappa locus, and then proceeds to the lambda locus, as described in both mouse and man[33-38]. This editing process can also take place at the heavy chain, termed heavy chain replacement[39, 40], and results in breaks within the CDR3, ultimately leaving a VH replacement fingerprint, and lengthening the overall HC CDR3 due to utilization of cryptic recombination signal sequences[41].

If receptor editing is unsuccessful, these high affinity, self-reactive clones are deleted from the repertoire during the early immature to immature B cell stage via an

arrest in development and eventual apoptosis after 2-3 days[42-44]. These central tolerance mechanisms occur before these cells enter circulation. In contrast, lower affinity self-reactive clones do not induce apoptosis, however, the clones do exhibit a shorter lifespan upon entering circulation compared to a non-self-reactive clone[45].

Upon emigration into the periphery, there is an additional tolerance checkpoint between the new emigrant stage and the mature naïve stage[46], presumably due to the interaction of B cell clones with circulating antigen, which in the absence of T cell help, leads to anergy and eventual deletion. Peripheral tolerance checkpoints will be discussed in more detail in a subsequent section of this chapter.

1.1.2 Germinal Center and Extrafollicular Responses to Antigen

There are several pathways by which a B cell that has interacted with antigen can develop into an antibody-secreting cell: the extrafollicular response, and the germinal center response. While there exists several antigen models utilizing influenza[47, 48], dextran[49] and others[50, 51], highlighting detailed analyses in mice injected with B cells specific for the antigen hen egg lysozyme (HEL)[52], we have a much clearer understanding of the secondary lymphoid processes that arise in the six days after a T cell-dependent immune response. First, there is a large burst of B cell proliferation within the follicle approximately day (D) 1-3 after immunization. Furthermore, it has been shown that on ~D3.5, there begins a shift of follicular HEL-specific B cells into the extrafolllicular splenic bridging channels, as well as in the follicle; the latter being suggestive of nascent germinal center formation. This shift into either extrafollicular sites or the germinal center is complete at ~D4. The extrafollicular response peaks at ~D5

and diminishes at ~D6, whereas the germinal center response persists beyond that time point.

Canonically, the germinal center (GC) is the site in which immunological memory is born. Circulating naïve B cells as well as antigen-experienced B cells home to secondary lymphoid organs via the afferent lymph system[53]. From there, antigen-inexperienced B cells can encounter antigen at the T:B cell border, and potentially enter the GC[54-56]. Most importantly, antigen-experienced B cells present peptide on its surface via major histocompatibility (MHC) molecules, which can then interact with the TCR on a cognate T cell, leading to the activation of both of these cells. Upon entering the GC post-antigen interaction, a B cell can acquire signals from both follicular dendritic cells (FDCs) and follicular T helper (T_{FH}) cells, namely antigen and B-cell activating factor (BAFF) from FDCs, and costimulation of surface receptors and IL-21 from T_{FH} cells[57]. In addition to the peptide-MHC (pMHC):TCR interaction, other interactions include receptor engagements such as CD40:CD40L, CD80/86:CD28 and, cytokines such as IL-4 and IL-10. These cell:cell interactions induce expression of activation-induced cytidine deaminase (AID), which promotes somatic hypermutation (SHM) and isotype class switching[58]. Upon differentiation into plasmablasts (PBs), these cells downregulate CXCR5, the receptor for CXCL13[59 60], and exit the secondary lymphoid organ via the efferent lymph system which is mediated by expression of CXCR4 and the chemokine CXCL12[61, 62]. Subsequently, these cells home to their niches located in the spleen and bone marrow[63]. In addition, GC B cells can give rise to memory B cells, which provide long-lasting memory to the organism and can react rapidly in the context of future infection with that same pathogen.

AID promotes SHM by deaminating cytidines with the Ig loci[58]. This occurs in the context of single-strand DNA alone, and induces a single-strand nick that promotes nucleotide substitution, increasing diversity of BCR sequence and through selection processes, leads to an increase in overall affinity for antigen. Mechanistically, AID functions by converting a cytidine to a uracil, which is then targeted by uracil-DNA glycosylase, which removes the uracil, leaving an abasic site which is then excised by apurinic/apyrimidinic endonuclease 1 to create the single strand nick, ultimately leading to transversion or transition mutations that alter the germline sequence of that BCR[64]. SHM, while in theory targets all cytosine residues, preferentially targets certain sequence hotspots, namely RGYW/DGYW[65, 66]. These AID mutation hotspots are concentrated in the CDRs of antibodies, and thus promote somatic mutation in the regions of the Ab directly involved in binding antigen to further diversify the B cell repertoire toward an antigen of interest[67].

In addition to SHM, expression of AID in B cells can also lead to class switch recombination (CSR). The IgM constant region in naïve B cells, for example, can be replaced with a downstream constant region **(Figure 1-3)** via a series of coordinated AID-mediated double-strand breaks located in switch regions upstream of each constant region[68, 69]. As a result, the intervening DNA between the two constant regions is deleted, and thus, CSR can only occur in a single direction along the chromosome. Ig isotypes differ in their characteristics related to the immune response. For example, IgG4 does not activate the classical complement pathway[70-72], while IgM, IgG1, and IgG3 can[73], and possess a myriad of roles within the context of an immune response. IgM and IgA Abs can form polymers that increase overall avidity for antigen; tetramers and dimers, respectively[74, 75]. In addition, IgG antibodies effectively cross the placenta, which

can provide protection of the fetus via maternal Abs, but can also induce complications in the context of Rh factor-positive fetus and an Rh factor-negative mother[76], as well as other examples.

The signals that govern which isotype the B cell switches to are multi-faceted. The cytokine environment plays a large role in mediating class switch to the various Ig isotypes, likely involving an interplay between IL-4 and IL-10, and others[77, 78]. CD40-stimulated B cells class switch first to IgG4, then IgE in the presence of IL-4 and IL-13[79-82]. IL-10 has been shown to induce both class switch and antibody secretion in CD40-stimulated B cells[83, 84], particularly of the IgG4 subclass[85-87]. In addition class switch may also be regulated by cell division, and has been described to occur during the third cell division in both mice and man[84, 88-90].

The qualities of the antigen:B cell interaction that define the differentiation path B cells enter are extremely complex. It is thought that the B cells that participate in the extrafollicular response are defined by abundance of antigen and affinity for that antigen[91]. In addition, the magnitude of the extrafollicular response is affinity-dependent, despite the observation of similar kinetics up to D5 post-immunization[52]. The kinetics of the extrafollicular response likely exist to produce a rapid production of high affinity antibodies that can mediate clearance in the short-term period post-infection. Meanwhile, additional high affinity clones can develop via the germinal center, and contribute to the longer-term protection of an individual after the extrafollicular response wanes.

The extrafollicular response has been canonically defined as a T cell-independent (TI) process; that is, the B cell does not require T cell interactions and/or

signals to differentiate into an antibody secreting cell. This has been very well described in the context of the marginal zone, wherein blood-borne particulate antigens first come into contact with B cells[92]. TI antigens are able to induce B cell differentiation due to antigen binding of both the BCR and Toll-like receptor (TLR). The fact that TI antibody responses occurs in the marginal zone is not surprising, as blood-borne bacteria that are brought to the spleen by circulating cells such as granulocytes and immature dendritic cells[93] would be capable of binding TLRs, thus circumventing the requirements of T cell help in inducing antibody secretion.

A challenge in the field is the understanding of the development events that leads to an immune response during infection and whether it is comprised of an antibody response derived from a TI B cell population or T cell-dependent (TD) population. The external signals required to induce B cells to secrete antibody has been demonstrated *in vitro*, as incubation of B cells with either anti-CD40 antibody with IL-4, or lipopolysaccharide (LPS) alone can induce proliferation and IgG[94]. Interestingly, in the absence of T cell interactions, such as in a mouse where Bcl6 has been conditionally ablated or even where CD40 has been knocked out, there is a population of low affinity, unmutated IgG against the immunizing antigen[95]. Patients with hyper IgM syndrome offer a unique view into the signals required in both the TI and TD immune response. Hyper IgM patients demonstrate unusually high levels of serum IgM and a complete lack of class-switched Ig. These patients subset into two main categories: one with defects in CD40 and others with deficiencies in AID. Interestingly, those patients with defects in CD40 harbor an $IgM^+IgD^+CD27^+$ population that demonstrates evidence of somatic mutation, which suggests that the TI developmental pathway of B cell differentiation can still lead to somatic mutation. In contrast, patients with deficiencies in AID exhibit GC

formation, but harbor an unmutated $IgM^+IgD^+CD27^+$ B cell population[92]. Therefore, one cannot outright conclude the origin of an antibody-secreting cell (ASC) based on solely Ig isotype and/or somatic mutation level. However, there does exist some surface markers that differentiate between a B cell that has entered the germinal center versus one that has not. CD73[96-98] and peanut agglutinin (PNA)[91, 99], for example, are thought to be expressed on the surface of B cells that have a germinal center origin.

1.1.3 Peripheral B cell Tolerance

In the previous sections, I have described two mechanisms of tolerance that occur in the bone marrow: receptor editing and deletion. In the periphery, as circulating B cells are exposed to a new set of antigens, additional mechanisms of peripheral tolerance prevent autoimmunity to these self-antigens, including anergy, somatic mutation, and even modification of BCRs via glycosylation[100].

In order to persist in the repertoire, a B cell must be able to compete for limiting survival factors such as BAFF, which is produced by stromal cells[101], as well as FDC, and binds to the BAFF receptor on the surface of B cells. BAFF interaction induces the expression of Bcl2[102], an anti-apoptotic molecule which counteracts Bcl6 interacting mediator of cell death (Bim) as well as BAD via the induction of PIM2 expression[103, 104]. B cell survival is therefore a balance in maintaining sufficient levels of anti-apoptotic factors and pro-apoptotic factors, wherein an excess in the latter direction leads to deletion. Peripheral tolerance helps to ensure that this imbalance in favor of pro-apoptotic molecules eliminates those cells that have the potential to react to self.

Tolerance mediated by a B cell-intrinsic pathway arises after peripheral antigen stimulation in the absence of T cell help[105]. Increased internalization of surface receptors and the inhibition of BCR transport out of the endoplasmic reticulum results in the overall downregulation of surface Ig expression[106], which has been observed in ~2.5% of circulating B cells[107]. Not surprisingly, these anergized cells are less able to induce signals downstream of the BCR, and thus require a larger threshold to overcome the constitutive, pro-apoptotic signaling by Bim[103]. Defects in the phosphatase pathways mediated by SHP1 (SH2-domain-containing protein tyrosine phosphatase 1) and SHIP (SH2-domain-containing inositol-5-phosphatase) result in increased susceptibility to producing autoAb[108, 109], highlighting the roles of these proteins in maintaining peripheral B cell tolerance.

Interestingly, studies probing these anergized cells have shown that this population, when subjected to IL-4 and CD40L or LPS *in vitro*, sill possessed the ability to become activated and differentiate into IgG secreting ASCs[94]. This suggests that in the context of a polyclonal B cell repertoire, these anergized B cells cannot compete for survival factors compared to other B cell populations. Localization of these anergized B cells may be playing a role in peripheral tolerance as well, as these cells are localized to the T/B cell border within the spleen[94], which may help sequester TLR ligands from this population, preventing activation *in vivo*.

The process of SHM allows for the generation of high-affinity memory B cell clones that provide long-lasting memory to the infected individual. However, this error-prone process, which occurs in the periphery, is likely to produce B cells that can bind self, as has been described in the context of polyreactive clones against charged antigens such as DNA and LPS[110].

Genetically, the VH4-34 gene segment offers two unique sequence characteristics that are relevant in the understanding peripheral selection. First, the germline VH4-34, due to an Ala–Val–Tyr motif located in the FWR1 of the VH4-34 gene segment, can mediate self-erythrocyte agglutination at low temperatures[111-114]. Mutations of this motif in human Abs has been observed to be more frequently mutated than what would be expected by chance[100, 112]. This suggests that the process of SHM may serendipitously select against HCs that maintain motifs promoting autoreactivity.

Secondly, the VH4-34 HC encodes a germline Asn–X–Ser/Thr sequon that promotes N-linked glycosylation of its CDR2. Glycosylation at this site precludes antigen binding, and has been in observed in the modulation of binding self-proteins[100, 115]. Thus, amino acid residues, arising from germline or even somatic mutation, that could promote glycosylation offer another means to abolish reactivity to self in addition to the canonical pathways of anergy and deletion.

While the VH4-34 heavy chain studies have shown that events in the germinal center can abolish self-reactivity, whether this selection process is active or passive is not clear. Robert Brink's group, as well as others[116], have developed elegant systems in which to test whether there is evidence of negative selection that prevents nascent autoreactive clones formed in the GC from contributing to the Ab repertoire[117]. This system required that:

1) a foreign antigen but not a self-antigen has the ability to bind a population of naïve B cells, and

2) the process of SHM permits a GC B cell to acquire cross-reactivity to both foreign and self.

Brink's system took advantage of HEL point mutants and the HEL-specific HyHEL10 heavy chain expressed as a transgene on SW_{HEL} B cells. The foreign antigen was a HEL^{3x} point mutant that was of intermediate affinity, and the self-antigen was a HEL^{4x} point mutant that was of such a low affinity that it did not induce activation of these SW_{HEL} B cells. However, a specific Y53D mutation increases the affinity of the transgenic BCR expressed SW_{HEL} B cells such that either HEL^{3x} or HEL^{4x} can activate this population.

Ubiquitous expression of HEL^{4x} prevents the development of Y53D mutated SW_{HEL} B cells upon challenge with HEL^{3x}. This effect was less pronounced when the level of self-antigen was reduced, and suggests that concentration of self-antigen in secondary lymphoid tissues can influence the level of negative selection imposed upon autoreactive clones. This is further supported by observations under high concentration of self-antigens, wherein FDCs presenting self-antigen can promote the deletion of GC B cells through Bim[105, 118].

In addition, by varying the promoter on which HEL^{4x} was expressed, Brink's group observed that in cases where the self-antigen is expressed in a tissue-specific manner, such as the liver or the kidney, self-reactive Y53D mutant SW_{HEL} B cells were able to persist, suggesting that for tissue-specific antigens, there is no tolerance checkpoint in the GC to prevent autoreactive clones from exiting the GC **(Figure 1-4)**. Thus, there must be other means to promote tolerance to tissue-specific antigens, such as those described above. This lack of germinal center tolerance to tissue-specific antigen is especially relevant in the context of PV, where Dsg3 expression is extremely restricted to stratified squamous epithelia and the thymus, and will be a small focus of my thesis.

1.1.4 Plasma Cells

The plasma cell (PC) is the terminal stage of B cell differentiation, and is the point at which this non-dividing B cell can secrete large amounts of soluble, antigen-specific Ab. As B cells differentiate into plasmablasts (PBs), and subsequently PCs, the transcription factors *Irf4*, *Xbp1,* and *Blimp1* are upregulated, with a concomitant reduction in the expression of *Pax5* and *Bcl6*[119], which are canonical transcription in mature naive B cells. Given the role of the plasmablast and plasma cells as sources of soluble antibody, which is on the order of 10,000 molecules per second per cell[120, 121], expression of Xbp1 allows for the induction of the unfolded protein response[122-124] to support the endoplasmic reticulum-based stress resulting from the production of large amounts of secreted antibody protein. PCs reside in the bone marrow niche, but can also be found in the spleen and inflamed tissues. In regards to the spleen, this migration is mediated by the migration towards CXCL9, 10 and 11 via surface expression of CXCR3[62, 125], and is thought to provide the maximal amount of antibody at the site(s) of infection. A similar form of trafficking has also been observed in the skin, mediated by the CCR6 ligand CCL20[126]. Upon resolution of infection, which will lead to the cessation of the production of survival signals, these tissue-resident PCs will die[119] due to loss of this niche.

Soluble antibodies have a half-life of approximately two weeks in the mouse[127] and ~twenty-five days in humans[128, 129]. Given this relatively short life of soluble Abs, this suggests one, if not more, of the following theories on how humoral immunity is maintained over a lifetime[119]:

1.) Homing and residence of LLPCs to survival niches

2.) Reconstitution of the LLPC pool with SLPCs that have undergone bystander activation

3.) Chronic antigenic stimulation offer constant regeneration of LLPC pool

It is unclear whether any of the above theories fully explain the maintenance of long-term serological memory, or whether reality is an amalgamation of all three. Studies conducted in humans after boost with tetanus toxoid have uncovered a slight rise in unrelated Abs[130]. Whether this is due to bystander activation of memory B cell clones, egress of ASCs from the bone marrow into circulation, or a combination of both, is not clear. However, what is clear is that the plasma cell niche cannot be infinite, and thus there must be some turnover within this niche to incorporate new antigen specificities while maintaining humoral immunity for the lifetime of the organism.

There are two main subsets of ASCs that are defined based on their ability to be long-lived. Short-lived plasma cells (SLPCs) are a rapidly renewing population that have a half-life of less than ten days[131], while it is thought that long-lived plasma cells (LLPCs) have a half-life of more than one hundred days and are thought to persist for the lifetime of the organism[132, 133]. SLPCs have approximately one week to migrate to a survival niche[119], which may be due to the gradual loss of the ability of this cell population to migrate toward CXCR3 and CXCR4 ligands over time, underscoring the tissue-resident characteristics of terminally-differentiated LLPCs[134-136]. Within the niche, various survival factors such as BAFF and A proliferation-inducing ligand (APRIL) likely contribute to the ability of these cells to persist. This is further supported by surface expression of BCMA and CD138 on PCs, both of which are receptors for APRIL[137, 138]; the former is also a receptor for BAFF. IL-6 can also extend the life of PCs *in vitro,* but whether this cytokine

plays a role *in vivo* is not as clear, as IL-6 deficient mice do not demonstrate defects in humoral immunity[139, 140].

Given the presence of both short-lived and long-lived plasma cells, the underlying cell populations that give rise to these two populations are still unclear. PBs can arise from any activated B cell: naïve, marginal zone, memory etc. However, the path to becoming a LLPC is not as straightforward as one may think, as not all SLPCs become LLPCs[141]. Transfer studies of bone marrow-resident B cell populations in mice suggest that both $CD44^{+}CD138^{+}$ and $CD44^{-}CD138^{-}$ B cells give rise to short-lived plasma cells, while the $CD44^{+}CD138^{-}$ and $CD44^{-}CD138^{+}$ B cell populations give rise to long-lived populations[131]. However, whether these bone-marrow resident cells are descendent from an extrafollicular or a germinal center B cell is still not clear.

Regardless of the source, ASCs contribute a very important role in ameliorating infection through the secretion for Ab. However, if these ASCs produce self-Abs, controlling this autoimmune reaction could be challenging, and my thesis research aims to understand the developmental events that lead to this unintended attack against self by using pemphigus vulgaris as a model disease to answer these questions.

1.2 Overview of Pemphigus Vulgaris

To answer the fundamental question of how autoimmunity develops, one must first find a suitable model disease to study. Pemphigus vulgaris (PV) is an autoimmune disease of the skin and oral cavity characterized by circulating serum antibodies (Abs) to the adhesion protein desmoglein 3 (Dsg3). While the association of autoantibodies in PV was first described in 1986[142], the autoantigen was first described five years later by

John Stanley's group[143]. PV is the most common form of pemphigus, which is a group of autoimmune diseases of the skin and oral mucosa characterized by intra-epidermal blistering, also known as acantholysis.

1.2.1 Distinguishing Pemphigus Vulgaris from other Pemphigus Subtypes

PV is subtype of the larger pemphigus group of autoimmune diseases. The different types of pemphigus are distinguished from each other largely based on clinical presentation and the self-antigen targeted in each subtype[144, 145]. In this regard, pemphigus vulgaris is diagnosed based on suprabasal blistering, or acantholysis, of the skin and/or oropharynx or a positive enzyme-linked immunoabsorption assay (ELISA) test against Dsg3, while pemphigus foliaceus (PF) is characterized by superficial blistering of the skin or a positive ELISA against Dsg1. Clinically, PV presents with blistering of the oral cavity and serum Ab titers against Dsg3, and in later disease, autoAbs against Dsg1 are also observed, and coincide with skin involvement. The underlying reasons behind this will be made clear later in this section.

In order to understand the different forms of pemphigus, one must first understand the function of Dsg in the epidermis. Dsg functions within the desmosome, which is a multi-molecular complex that connects keratinocytes to each other in stratified squamous epithelia; namely the epidermis and the oral mucosa. Similar to all cadherins, the extracellular (EC) region of Dsg is divided into five regions, termed EC1-5[146] **(Figure 1-5 A)**. EC1 and EC2 being the most distal from the keratinocyte surface, have been shown to mediate both *trans* and *cis* interactions with neighboring keratinocytes, wherein *trans* interactions are with neighboring keratinocytes and *cis* interactions are with

desmosomal molecules on the same keratinocyte cell surface **(Figure 1-5 B)**[147, 148]. *Trans* interactions are calcium-dependent[149, 150], relying on Dsg binding to three calcium ions in pockets between the EC domains[151, 152]. This dependence on calcium for rigidity and cadherin interactions is a characteristic of all cadherin family members[153]. The extracellular *trans* interactions on one end and interactions with intracellular keratin filaments on the other provides the desmosome with the ability to resist to mechanical stresses typically encountered within the context of stratified squamous epithelia. Within the context of PV, the presence of Dsg3-specific autoAb are thought to interfere with these adhesion properties of Dsg3, ultimately leading to acantholysis.

Dsg1, the self-antigen in PF, is a close family member to Dsg3 and is also a component of the desmosome. There is evidence of homology between Dsg1 and Dsg3, roughly 73% identity within EC1, and this homology decreases with each subsequent EC domain[143]. It has been shown that Dsg3 and Dsg1 expression within the epidermis is inversely related; Dsg3 expression decreases with further differentiation of keratinocytes, while Dsg1 expression increases with further differentiation[154-156]. As keratinocytes become fully differentiated in the superficial layers of epidermis, the compensation theory was born to explain the differences in the clinical presentation of PF and PV patients[157, 158] in terms of blister occurrence within the epidermis. The compensation theory posits that while there exists antibody against Dsg3, if Dsg1 is concomitantly expressed within that same layer of epidermis, the loss of Dsg3-mediated adhesion is rescued by the presence of Dsg1 **(Figure 1-6)**. Therefore, only in layers of epidermis where there is no Dsg1 to compensate would we observe a blister, and vice versa for autoAb against Dsg1. This is supported by data in Dsg3-deficient mice that demonstrate a more severe blistering phenotype when injected with anti-Dsg1 serum from a PF

patient[158]. This compensation theory has been proven in transgenic mouse models where Dsg3 or Dsg1 was expressed throughout the epidermis and protected against both blister formation[159, 160] and telogen hair loss[161], the latter of which was observed in an early mouse model of PV[162].

1.2.2 Mechanisms of Disease Pathogenesis

Direct binding of autoAb to the EC1 and EC2 domains of Dsg3 can directly interfere with calcium-sensitive *trans* interactions between two keratinocytes via steric hindrance[163, 164]. There have also been several descriptions of autoAb binding to domains such as EC4 [165-167], which are not known to be responsible in mediating the *trans* interactions, suggesting additional mechanisms of autoAb-mediated inhibition of Dsg3 adhesion. These differences in Dsg3-epitope binding distinguish pathogenic Abs from non-pathogenic; that is, monoclonal Abs that can cause a blister in various assays, versus those that cannot. Interestingly, it has been shown that incubation of multiple non-pathogenic Abs can induce acantholytic blisters[168], suggesting that these non-pathogenic Abs still play important roles in disease pathogenesis. In addition, whether anti-Dsg3 Abs are pathogenic or non-pathogenic is irrelevant given that the fact remains that these Abs still react against self, and that I ultimately want to understand tolerance towards self-antigens as a whole.

Other than direct inhibition of Dsg3 adhesion, an additional mechanism of auto-Ab mediated interference is the induction of Dsg3 internalization, which is thought to be caused by initiation of various signaling pathways downstream of autoAb binding to Dsg3[169-171]. This internalization is specific, as desmocollin 3, another desmosomal

protein, is not internalized upon incubation with anti-Dsg3 Ab[172]. Time lapse electron microscopy experiments have also demonstrated that autoAb binding inhibits the ability of Dsg3 to enter simple clusters that occur before Dsg3 enters the desmosome due to internalization of Dsg3 into endosomes[172, 173]. These interferences mediated by Dsg3 autoAb on both Dsg3 formation and function then leads to the characteristic separation of the suprabasal layers of epidermis, otherwise known as acantholysis.

There does exist evidence detailing the role of apoptosis in PV; although it is unclear whether this is a primary or secondary response in disease pathogenesis. It has been shown that some acantholytic blisters contain apoptotic keratinocytes. In accordance with this observation, expression of molecules involved in the apoptosis death pathway, such as Fas ligand and its receptor FasR, p53, and Annexin V, are also observed within an acantholytic blister[174]. Incubation of keratinocytes with PV-IgG induced the cleavage of caspase 1, 3, and 8, further supporting a potential role for apoptotic cell death in acantholysis. Interestingly, treatment with either PV-IgG or anti-FasR antibodies both result in the formation of the death-induced signaling complex. Despite evidence suggesting that acantholysis is not completely apoptosis-dependent, as acantholysis was observed even in the presence of caspase 8 inhibitors[175], collectively, the studies described above underscore a potential role for apoptosis in the clinical presentation of this tissue-specific autoimmune disease.

1.2.3 Detailing the role of IgG isotype in Pemphigus Vulgaris

Looking at the autoAbs more closely, it has been described that there is a correlation of serum IgG4 Ab titers and disease severity[176-179]. Interestingly, healthy

relatives of patients with PV demonstrate non-pathogenic Dsg3-specific IgG1 that does not induce blistering, while active patients demonstrate both Dsg3-specific IgG1 and IgG4[180]. IgG4 is typically observed in patients undergoing desensitization therapy for chronic allergies[181, 182], as well as in beekeepers[183]. The latter observation is thought to arise from chronic injections of bee venom due to work-related risk factors. The IgG4 constant region is inhibitory; that is, IgG4 cannot induce complement activation, crosslink antigen, or form immune complexes[70-72], and thus IgG4 can act in an anti-inflammatory role. Given the context of IgG4 development during the immune response, it is likely that IgG4 results as a consequence of chronic antigen interactions, perhaps acting in a protective pathway to inhibit further activation of other immune cells in the future.

1.2.4 Interleukin 10's Role in Pemphigus Vulgaris

Interleukin (IL)-10's ability to induce IgG4 class switch[85-87] suggests a pathogenic role for this cytokine in PV, but various studies have reported both protective and detrimental effects of IL-10[184]. IL-10, as well as IL-4, is expressed at a higher level in pathogenic versus non-pathogenic anti-Dsg3 T cell clones. However, exogenous IL-10 administration to primed B cells *in vitro* does not induce anti-Dsg3 secretion, nor does *in vivo* blockade of IL-10 affect disease incidence in the murine model of PV[185]. In contrast, mice deficient in IL-10 demonstrate increased susceptibility to acantholysis after passive transfer of PV patient sera, which can be prevented upon IL-10 administration after passive transfer of PV patient sera in wildtype mice[186]. It has been shown that IL-10 can also inhibit the p38/MAPKAP-kinase 2 pathway[187], which can protect against acantholysis by regulating the endocytosis of Dsg3 upon anti-Dsg3 antibody interactions[188-191]. Taken as a whole, these data in murine systems underscore a role for

IL-10 post Dsg3:antibody interactions rather than a role for IL-10 in the development of these anti-Dsg3 antibodies.

In humans, IL-10 in the context of active PV seems to promote disease, perhaps through the differentiation of Dsg3-reactive B cells preferentially into IgG4 ASCs due to the lack of Dsg3-reactive Tregs[192], whose role will be discussed in further detail below. In addition, an increased level of IL-10+ B regulatory cells after treatment with rituximab to ablate the B cell repertoire in these patients is associated with complete remission[193], and may suggest an inhibitory role for IL-10.

Overall, the myriad roles of IL-10 in the immune response may point to context-dependent roles for IL-10 in PV based on disease stage. In addition, studies in systemic lupus erythematosus and rheumatoid arthritis have also revealed disparate roles for IL-10[184], suggesting that a context-dependent role of IL-10 may also occur in other autoimmune disorders in addition to PV.

1.2.5 The Role of the T cell in Pemphigus Vulgaris

Canonically, T cells are required for B cells to become ASCs. Thus, the role of the T cell in PV cannot be ignored. It has been shown that Dsg3 is expressed under the *Aire* promoter by medullary thymic epithelial cells[194], which suggests that Dsg3-reactive T cells should be eliminated from the repertoire. In addition, Dsg3 was not shown to be expressed in extrathymic *Aire*-expressing cells[195], which are thought to mediate negative selection of autoreactive B cells in the periphery.

Previous reported have described strong associations of certain MHC haplotypes in PV, namely HLA-DRB*0402 in Jewish populations[196, 197] and HLA-DQB*0503 in non-Jewish populations[198, 199], the former of which is rare in the general population, suggesting a role for MHC and antigen presentation in disease. Structural studies detailing these PV susceptibility alleles demonstrate interactions between a positively charged Dsg3 peptide with a negative charge in the P4 pocket of the MHC molecule[200], underscoring the reason for their overrepresentation in patients with PV.

Genome-wide association studies (GWAS) have uncovered differentially-expressed gene (DEG) "signatures" for various stages of disease, such as "control", "activity" and "disease"[201], although these GWAS do not discern between whether these DEGs arise as a causative or secondary effect of disease pathogenesis. In addition to the HLA susceptibility alleles, there is an association of PV and increased expression of the ST18 gene, which has a role in the regulation of apoptosis and inflammation[202]. It should be noted that this same gene is also upregulated in the context of psoriasis, and thus it is unclear whether this upregulation of ST18 expression precedes disease or whether ST18 is simply a marker for skin inflammation as a whole.

Recently, an increase in the number of circulating follicular helper T (T_{FH}) cells has been observed in PV patients compared to healthy individuals and patients with myasthenia gravis, a distinct neurological autoAb-mediated autoimmune disease[203]. This increased frequency of T_{FH} appears to be non-specific to PV, as similar observations have also been described in systemic lupus erythematosus[204] and rheumatoid arthritis[205]. However, it is possible that increased numbers of T_{FH} in PV patients could be mediating the ability of these Dsg3-reactive B cells to become ASC.

A highly intriguing observation in PV patients, as well as healthy individuals expressing the PV HLA susceptibility alleles, is that both of these populations demonstrate a low but statistically significant population of circulating Dsg3-reactive T cells compared to HLA-unmatched controls[206-208], suggesting that T cell tolerance in the population may be suboptimal, perhaps due to presentation of certain epitopes in the context of the aforementioned MHC molecules that associate with PV. Somewhat unsurprisingly, the detection of an anti-Dsg3 Ab-secreting B cell population by ELISPOT requires the presence of T cells[209] further supporting a loss of tolerance in the T cell compartment that contributes to disease pathogenesis. Interestingly, this loss of T cell tolerance can occur in a single T cell clone, as a single potent Dsg3-reactive T cell can induce a polyclonal Ab response in the murine model of PV[210]. Clearly, the immune system, if relaxed, perhaps in the context of a rampant infection, could provoke a formidable autoimmune response to Dsg3 if kept unchecked.

Michael Hertl's group also characterized a population of Dsg3-reactive Tregs in healthy relatives of patients with PV, as defined by expression of CD25, glucocorticoid-induced TNFR family-related receptor, and the presence of FoxP3 mRNA[192, 211]. These Tregs were able to suppress T helper cells through a contact-independent manner, utilizing soluble anti-inflammatory cytokines including IL-10 and TGFβ. Strikingly, this Treg population was severely reduced in the PV patients studied compared to healthy, HLA-matched controls. Inhibition of FoxP3 via antisense oligonucleotides converts the Dsg3-specific Tregs into Th2 cells that can no longer suppress, but can proliferate and secrete cytokine in response to incubation with Dsg3 protein[212]. The lack of a suppressive T cell population to cull the anti-Dsg3 autoimmune response leads me to suggest an active mechanism of suppression through the secretion of the

immunosuppressive cytokines IL-10 and TGFβ by Dsg3-specific Tregs, which will be discussed in Chapter 4.

A fascinating observation in PV is the fact that anti-Dsg3 autoAbs are necessary and sufficient to confer the blistering phenotype observed in patients in both neonatal mice and *ex vivo* human skin [213-215, 215]. In addition, this phenotype is independent of the constant region of the autoAb[216, 217], suggesting that is the direct binding of these autoAbs to Dsg3 that results in acantholysis. Because of the very clear role for autoAb in PV, I wanted to better understand how the B cells that secrete these autoAb arise, and why they persist within a patient to induce disease. This is the focus of my thesis, and will be discussed in subsequent chapters.

1.3 Overview of Rotavirus

Rotavirus is a segmented dsRNA virus of the *Reoviridae* family[218]. It is the most common cause of diarrhea in the world[219], and is 95% penetrant by age 5[220]. It is responsible for 2 million hospitalizations and over 600,000 deaths annually across the globe[221], the majority occurring on the Indian subcontinent.

Rotavirus infections peak during the winter season[221]. Rotavirus is transmitted through the fecal-oral route, and potentially through respiratory droplets[222], as it can infect cells of both the gastrointestinal and respiratory tract[223-225]. A high prevalence of anti-rotavirus antibodies throughout life suggests re-infection, and in some cases, are likely subclinical, as a percentage of rotavirus-infected adults can be asymptomatic[226, 227].

The eleven segments of the rotavirus genome are encapsulated by a triple-layered icosahedral virion. The eleven segments encode six structural proteins (VP1 through VP4, VP6, and VP7), and six nonstructural proteins (NSP 1 through NSP6)[228].

The outermost layer is comprised of VP4 and VP7, while the intermediate layer is comprised of 260 VP6 trimers[229-231]. Lastly, the innermost layer is comprised of VP2, which surrounds the transcriptional machinery, namely VP1 and VP3, the former being the RNA-dependent RNA polymerase[232, 233]. As a triple layered particle (TLP), rotavirus is transcriptionally inactive. Upon internalization of the TLP into the host cell via VP7 binding to the host cell surface, the outermost layer is shed, allowing the double-layered particle (DLP) to become transcriptionally active[229, 234, 235]. Mechanistically, the DLP extrudes RNA through a central pore formed by a VP6 trimer for translation by the host cell. There are twelve of these egress points, termed Type I channels, on the surface of the DLP[232].

1.3.1 Detailing the antibody response to rotavirus

While antibodies to VP4 and VP7 are neutralizing, levels of neutralizing Ab against VP4 and VP7 do not always correlate with protection[236, 237]. In contrast, antibodies to VP6, which is the most antigenic protein in the virion[238, 239], are non-neutralizing in that they cannot prevent endocytosis of the rotavirus virion[240]. In addition, the VP6 protein is both the most antigenic and the most conserved across all strains of rotavirus[241] **(Figure 1-7)**. In fact, it determines which group each rotavirus strain belongs to; Group A rotaviruses are the main cause of gastroenteritis in humans[233].

Given, the non-neutralizing properties of anti-VP6 antibodies, it is thought that anti-VP6 IgA Abs function during transcytosis of the molecules across the intestinal membrane[242-245], interfering with virion replication through several distinct mechanisms. This includes steric hindrance of the RNA extrusion pore within the Type 1 channel, as well as altering the conformation of the Type I channel, both of which can inhibit RNA extrusion from the DLP and therefore, replication of the rotavirus particle within the host cell[245-247]. However, not all anti-VP6 Abs are able to inhibit rotavirus transcription[246-248], and the functional roles of these non-inhibiting anti-VP6 Abs are not fully understood. Anti-VP6 IgG as a whole may function to promote opsonization, however, the role of anti-VP6 IgG in viral clearance has not been fully characterized. It has been shown that mucosal immunization of mice, either intranasally or orally, induced serum anti-VP6 IgG responses[249]. In addition, immunization with VP6 induces protection against rotavirus in several different animal models[250-254], and that this protection post-VP6 immunization can be achieved in the absence of IgA[255] and even B cells[249]. Thus, the role of VP6 in promoting an immune response seems to be complex and not restricted to a purely B cell response.

It has been shown that rotavirus infection induces polyclonal activation of mature naïve B cells[256] within the secondary lymph nodes and Peyer's patches, leading to the production of rotavirus-specific IgM as early as three days post-infection. Interestingly, this early polyclonal activation is independent of T cells, as a T cell-deficient mouse exhibits a similar phenotype, suggesting a potential TI pathway of IgM production. The rotavirus protein NSP4, which acts as an enterotoxin[257, 258], has been shown to be able to induce the activation of macrophages and subsequent secretion of proinflammatory cytokines through TLR2[259], and perhaps this same mechanism of cell activation holds

true in the B cell compartment as well. This would not be surprising, as strong signals from TLRs binding cognate ligands can bypass the antigen specificity requirement to activate B cells via the BCR[260-262].

In characterizing this polyclonal B cell activation more closely, it was discovered that only TLPs were able to induce polyclonal activation, and this activation was achieved through the VP7 protein expressed on the surface of the TLP[263]. How VP7 initiates this polyclonal response is uncertain, but the contribution of plasmacytoid dendritic cells (pDCs) in rotavirus infection likely plays a role, as loss of this population in mice increases viral titers with a concomitant decrease in serum rotavirus antibody titers[264]. Polyclonal B cell activation could arise from bystander activation mediated by the secretion of type I interferon from pDCs activated via TLR7[265], which occurs after the decapsidation of the TLP within the endosome to expose viral dsRNA after internalization of the virion.

Genetic characterization of the circulating B cell response to the VP6 protein was first reported in 2003, where James Crowe's group compared the anti- VP6 immune response in infants and adults, showing a bias towards the VH1-46 gene segment in both patient populations. This was in contrast to randomly selected B cells, which demonstrated a VH3 family bias[266]. The level of somatic mutations in VP6-specific Abs markedly differed between these two patient populations by four-fold, yet half of the mutations observed were located in AID hotspot motifs[267].

Poor Ab responses to rotavirus in infants may also be related to the passive transfer of maternal IgG or IgA to the fetus, which occurs through the placenta and breast milk, respectively[268]. Indeed, studies in pigs[269] have recapitulated the

phenomenon that the presence of anti-rotavirus maternal IgG reduces rotavirus-related symptoms, but also impairs the ASC response post-infection. This is not specific to rotavirus either; protection in infants administered a trivalent inactivated influenza vaccine was markedly lower when maternal Abs were present[270], and thus may play some role, along with lower rates of somatic mutation, in the less than ideal protection rate of ~80% for the current rotavirus vaccines[271-273].

The VH1-46 restriction observed in the systemic anti-VP6 Ab repertoires of both infants and adults is striking, as it suggests that VH1-46 Abs may have an inherent advantage within the context of a rotavirus VP6 response due to their germline BCR sequences. This was further supported by studies analyzing the gut-specific, $\alpha_4\beta_7$-expressing IgD^- population of VP6-specific clones, where VH1-46 was again dominant, and demonstrated a markedly lower somatic mutation frequency compared to randomly selected, $\alpha_4\beta_7^+$ IgD^- memory B cells (0.3 versus 5.7%, p= 0.002)[274].

This VH1-46 bias in infants was further attributed to the $CD5^-$ B cell population, as the $CD5^+$ subset of rotavirus-specific B cells was dominated by VH3-23[275], which is thought to be more prevalent in infants[276, 277]. Both populations, however, demonstrated low somatic mutation frequencies, suggesting that a lack of somatic mutation is a facet of all B cell subsets in infants.

Extensive studies have characterized the anti-VP6 B cell response in adults based on subset. This strong VH1-46 gene restriction in the anti-VP6 Ab repertoire of acutely infected rotavirus patients is most apparent in the IgD^+CD27^- subset, and shrinks with the upregulation of surface CD27 and reduction of surface IgD, resulting in a decrease from >25% of the repertoire in the IgD^+CD27^- subset, to roughly 8% of both the

IgD^+CD27^+ and IgD^-CD27^+ anti-VP6 repertoire. Within the IgD^-CD27^+ population, VH1-02 becomes the most represented VH gene segment at about 10%[278]. It should be noted that VH1-46 representation in the control populations in these subsets are significantly lower, but this diminishing utilization of VH1-46 is still apparent in the rotavirus-infected cohort, as it is not observed in randomly selected B cells. This may be due to efforts within the GC in regard to affinity maturation that likely diversifies the anti-VP6 response, resulting in VH1-46 being overtaken as the dominant VH gene utilized in the IgD^-CD27^+ population of B cells. However, the bias of VH1-46 observed in the IgD^+CD27^- alludes to VH1-46+ B cells acting as a primary population in the initial immune response to VP6 early on during the course of infection, and may suggest a mechanism by which the body generates a full, diverse immune response against rotavirus.

Detailed structure-function studies of the somatic mutations in two VP6-specific VH1-46 mAbs have described a dependence on mutations located both in and around the heavy chain CDR2 for binding to VP6[266, 279] **(Figure 1-8)**. Furthermore, this dependence on the heavy chain was still apparent in the context of a chimeric antibody lacking a heavy chain CDR3, nor the physiologic light chain that arose from that same B cell that exhibited only a 10-fold reduction in affinity for VP6[279]. Given the role of the CDRs in defining specificity, these findings are somewhat unsurprising, but the fact that these distinct VH1-46 mAbs have a particular reliance on the heavy chain CDR2 for antigen affinity, which is germline-encoded by the VH gene segment, underscores a reason for the inherent biases for VH1-46 representation within the anti-VP6 Ab repertoire.

How these anti-VP6, VH1-46 Abs mediate protection have been described above. It should be emphasized that the two VH1-46 anti-VP6 mAbs that have been

thoroughly characterized show juxtaposing ability to inhibit transcription via steric hindrance of the RNA extrusion pore, in that one VH1-46 can[245, 279], and the other cannot[248, 279]. Regardless of their ability to inhibit rotavirus replication, their role in rotavirus is still relevant, and may contribute to the VH1-46 Ab response in pemphigus vulgaris. This idea will be tested in Chapter 3.

1.4 Experimental Questions

1. Does there exist any shared characteristics in the anti-Dsg3 Ab repertoires in unrelated patients, and if so, why?

2. Can we identify a potential route of how anti-Dsg3 autoAb arise, perhaps through shared genetic characteristics in the antibody responses between a self-antigen and a foreign antigen?

1.5 Figures

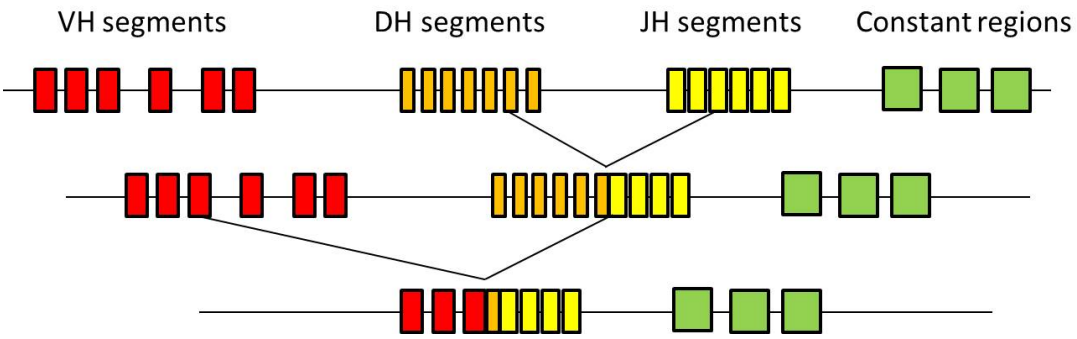


Figure 1-1. Overview of VDJ recombination

Simplified depiction of VDJ recombination at the immunoglobulin heavy chain locus. Recombinase-activating gene (RAG) 1 and 2 initiate a DNA recombination event, bringing together one of the 23 and one of the six DH and JH segments, respectively. A subsequent DNA recombination event joins one of the 38-46 functional VH gene segments to this DH-JH join. This nascent heavy chain is then subjected to functional testing upon pairing with a surrogate light chain.

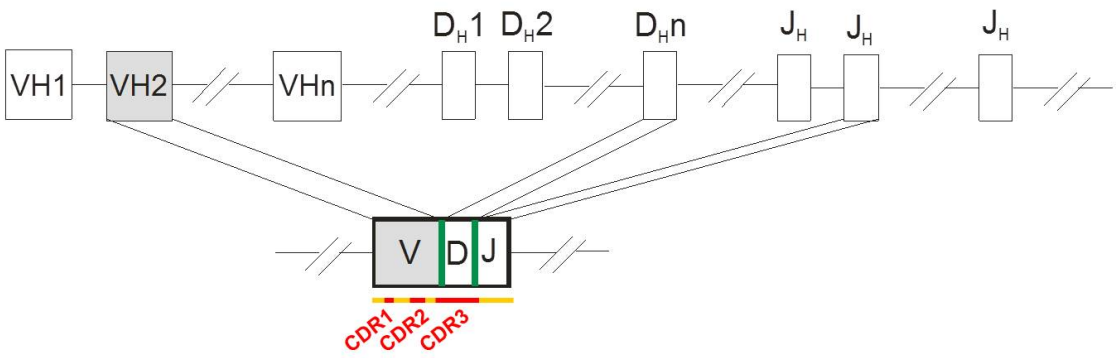


Figure 1-2. Schematic of a rearranged immunoglobulin heavy chain

Complementarity determining regions (CDRs) are highlighted in red. Framework regions are highlighted in yellow. The CDR1 and CDR2 are encoded entirely by the germline sequence of the VH gene segment; the CDR3 spans the VDJ junction.

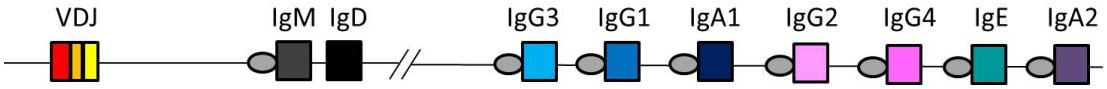


Figure 1-3. Diagram of the immunoglobulin heavy chain constant regions

A simplified schematic of the constant regions downstream of the immunoglobulin heavy chain locus. Switch regions are indicated by a grey oval, which mediates class switch by activation-induced cytidine deaminase. Due to deletion of intervening DNA between two switch regions, class switch can only occur in one direction; from left to right.

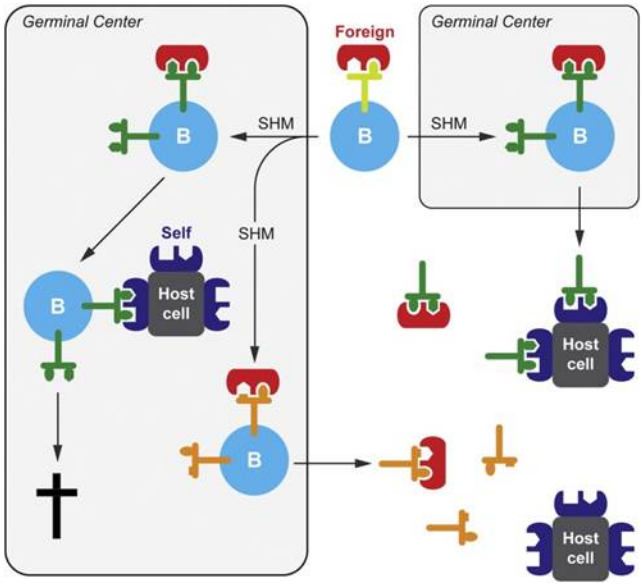


Figure 1-4. The role of antigen in peripheral selection

Robert Brink and colleagues determined that anatomical expression of self-antigen plays a role in negative selection within the germinal center, wherein expression of self-antigen within secondary lymphoid tissue promotes the deletion of self-reactive B cells (left). In contrast, B cells specific for self-antigens that are tissue-restricted, and thus not expressed in secondary lymphoid tissue, are not subject to deletion (right).

Source: Chan,T.D. *et al.* Elimination of germinal-center-derived self-reactive B cells is governed by the location and concentration of self-antigen. *Immunity.* 37, 893-904 (2012).

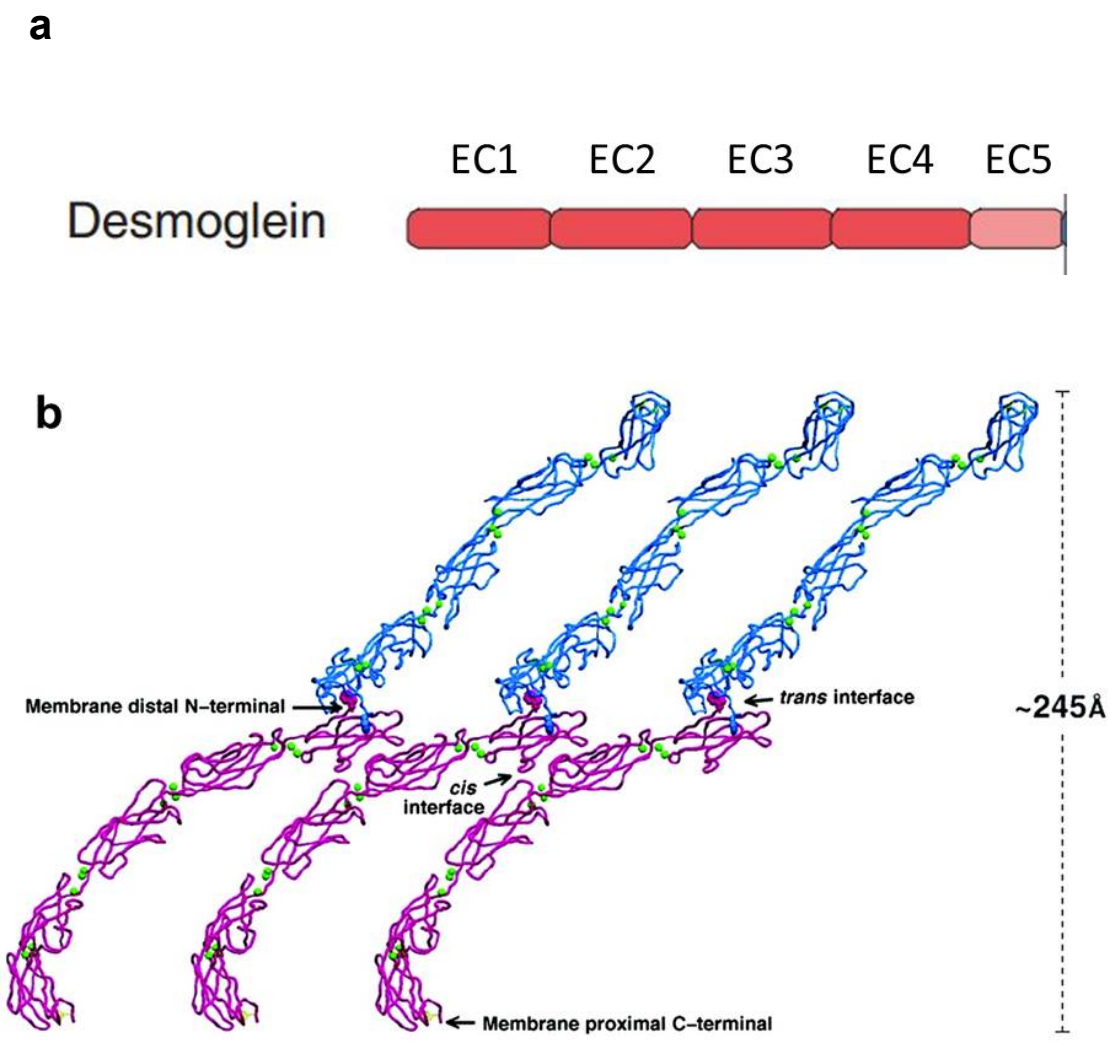


Figure 1-5. Structure of desmoglein, a member of the cadherin family

(a) Schematic representation of the extracellular (EC) domains of desmoglein.

Adapted from Ishii,K. Identification of desmoglein as a cadherin and analysis of desmoglein domain structure. J. Invest Dermatol. 127, E6-E7 (2007).

(b) Crystal structures of C-cadherin, highlighting both *trans* and *cis* interactions that would occur between two neighboring cells.

Source:

Boggon,T.J. *et al.* C-cadherin ectodomain structure and implications for cell adhesion mechanisms. *Science* **296**, 1308-1313 (2002).

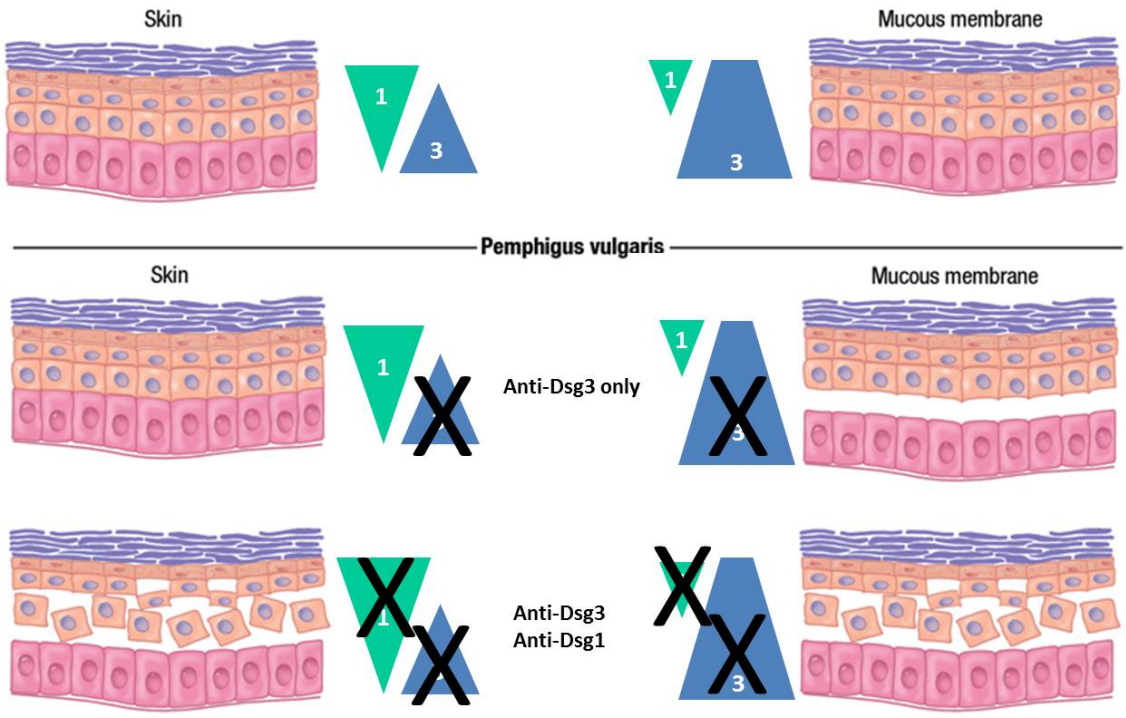


Figure 1-6. Overview of the desmoglein compensation theory

Expression of desmoglein (Dsg)1 and Dsg3 in the epithelium is inversely-related to one another (green and blue triangles, respectively). As a result, the layers in which blisters are apparent differ depending on disease stage. When anti-Dsg3 antibodies (Abs) are detected, pemphigus vulgaris (PV) patients present with blisters in the oral cavity, as Dsg1 is not fully expressed throughout the epidermis of the mucous membrane, and cannot compensate for the loss of Dsg3. This is in contrast to the skin, where Dsg1 can compensate for the loss of Dsg3, and thus patients do no present with blisters on the skin. As disease progresses to include anti-Dsg1 Abs, PV patients present with blisters of the skin and the oral cavity due to loss of both Dsg1- and Dsg3-mediated adhesion.

Adapted from Wolff K, Goldsmith LA, Katz SI, Gilchrest BA, Paller AS, Leffell DJ: *Fitzpatrick's Dermatology in General Medicine,* 7th Edition: http://www.accessmedicine.com

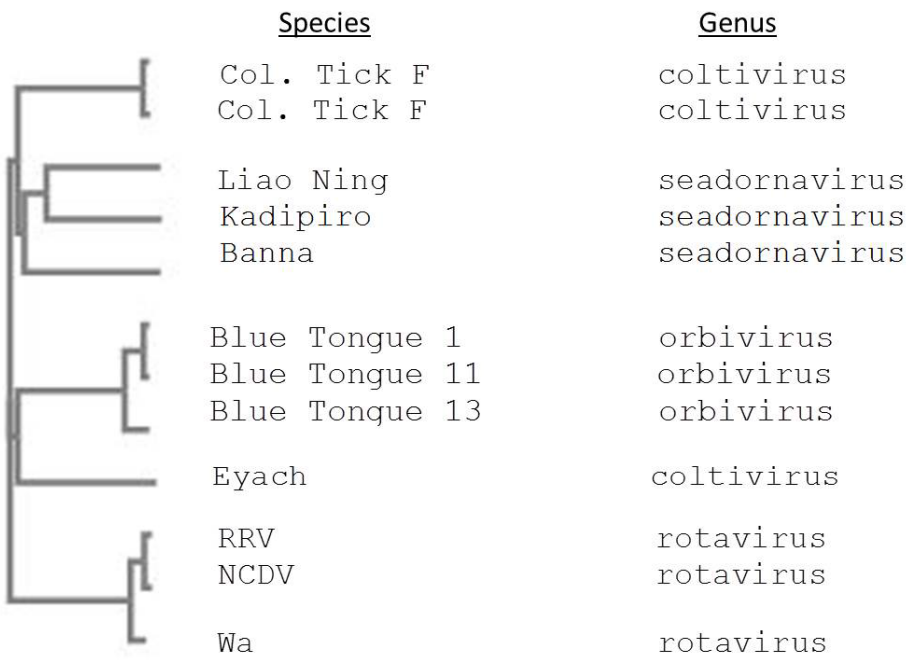


Figure 1-7. Diversity of VP6 within the *Reoviridae* family

Rotavirus belongs to the dsRNA virus family *Reoviridae.* VP6 amino acid sequences from selected genera of *Reoviridae* were aligned using ClustalW2. The three selected VP6 sequences, taken from three strains that have either been isolated from humans or been used to vaccinate humans, cluster away from other *Reoviridae* genera.

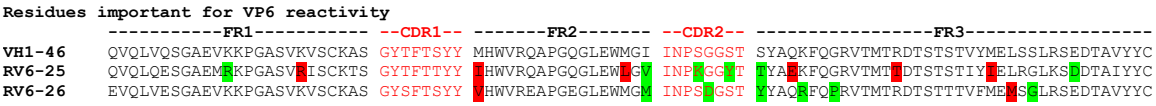


Figure 1-8. Overview of amino acid residues relevant in two VH1-46 antibodies specific to VP6

Residues that increase affinity for VP6 are highlighted in green, and residues that decrease affinity are highlighted in red. Green residues are enriched in and around the heavy chain complementarity determining region (CDR) 2.

Sources:
Weitkamp,J.H. *et al.* Infant and adult human B cell responses to rotavirus share common immunodominant variable gene repertoires. *J Immunol.* 171, 4680-4688 (2003).

Kallewaard,N.L. *et al.* Functional maturation of the human antibody response to rotavirus. *J*
Immunol. 180, 3980-3989 (2008)

CHAPTER 2: COMMON HUMORAL RESPONSES TO DESMOGLEIN 3 AS EVIDENCED BY SHARED VH1-46 GENE USAGE

2.1 Abstract

Pemphigus vulgaris (PV) is a potentially fatal autoimmune disease in humans characterized by suprabasal blistering of the skin and oral mucosa due to autoantibodies (autoAb) against desmoglein 3 (Dsg3), a transmembrane desmosomal cadherin that mediates keratinocyte adhesion in the epidermis. AutoAb repertoire cloning from four PV patients with active disease using phage display and heterohybridoma technologies reveals shared VH gene usage among Dsg3 autoAbs, with pathogenic VH1-46 Abs identified from all four patients. Sequence analysis indicates a population of VH1-46 Abs demonstrating less replacement mutations than non-VH1-46 counterparts. To determine the role of somatic hypermutation in the development of Dsg3 autoreactivity, we reverted somatically mutated Abs to their germline sequences and tested these Abs for their ability to bind Dsg3. Three of five VH1-46 germline Abs bound to Dsg3, while zero of five non-VH1-46 germline Ab did not. Site-directed mutagenesis studies indicate that acidic amino acid residues in the VH1-46 CDRs are necessary and sufficient for Dsg3 reactivity. Our data demonstrate that a subset of PV autoAb utilizing VH1-46 are able to bind Dsg3 without somatic mutation, suggesting that a population of naïve B cells in these patients were autoreactive upon entering circulation. In contrast, another subset of Abs require somatic mutations to become Dsg3 autoreactive, likely as a result of the germinal center reaction. This work suggests two separate development pathways leading to Dsg3 reactive autoAb that be shared across unrelated patients.

2.2 Introduction

Antibody (Ab) repertoires in humans are diverse and can form as many as 100 billion unique specificities[28, 29]. However, the process of forming the Ab repertoire is certain to create autoreactive B cells that must be deleted or remain dormant in order to prevent autoimmunity. Autoimmunity affects 8% of the U.S. population[1], and is the third most prevalent category of disease. The relatively high prevalence of disease demonstrates the fallibility of the human immune system, with errors leading to potentially self-destructive consequences. In order to better understand and treat autoimmune diseases, we must first understand the mechanisms by which autoreactive clones are created and why the immune system allows these clones to persist and ultimately lead to autoimmunity.

Pemphigus vulgaris (PV) is a tissue-specific autoimmune disease characterized by autoAbs to the epidermal protein desmoglein 3 (Dsg3). Dsg3 autoAbs are necessary and sufficient to cause pathognomonic blisters in several models of PV[213-215]. Prior studies have underscored the role of T cells in PV, wherein >95% of PV patients express the human leukocyte antigen (HLA) alleles DRB1*0402 and DQB1*0503[197, 280], signifying a genetic predisposition to PV. However, the majority of individuals expressing these HLA alleles do not go on to develop PV[280], indicating that HLA haplotype is not sufficient in the development of PV. Dsg3 is expressed under the Aire promoter in the thymus, which likely induces deletion of Dsg3-autoreactive T cells[195]. However, studies have shown that unaffected carriers of HLA-susceptibility alleles demonstrate a low frequency of circulating Dsg3-reactive T cells, similar to frequencies observed in PV patients[208]. In

addition, healthy carriers of HLA-susceptibility alleles demonstrate higher frequencies of regulatory T cells[192] in comparison to patients with PV, suggesting that T effector and T regulatory cells may participate in the regulation of tolerance to Dsg3.

Currently, while there are some descriptions of the B cell repertoire in PV [193, 281-283], none are comprehensive and effectively probe into the tolerance checkpoints that are overtaken Dsg3-autoreactive B cells. To form a B cell receptor, a B cell clone will undergo V(D)J recombination rearranges VH, DH, and JH gene segments to encode a diverse repertoire of heavy chains, which pairs with functional light chains derived from a parallel mechanism[8, 9]. Upon interaction with its antigen in the periphery, a B cell can then undergo somatic mutation, a process by which activation-induced cytidine deaminase and DNA polymerase η introduce DNA mutations into the B cell receptor sequence to increase affinity for antigen[284].

In order to understand the mechanisms by which Dsg3 Abs arise, our lab has cloned the Dsg3-specific Ab repertoires of four PV patients with active disease. Across all four patients, we isolate anti-Dsg3 Abs that utilize the VH1-46 gene segment. Furthermore, we hypothesize that these VH1-46 Abs may be physiologically more fit to bind Dsg3 due to the requirement of zero to very few somatic mutations to bind Dsg3, which may explain their presence in the anti-Dsg3 Ab repertoires of four different patients.

2.3 Results

To determine whether there exists an equivalent B cell tolerance mechanism to eliminate Dsg3-reactive clones from the repertoire as described in Chapter 1, we

conducted qPCR to detect the presence of Dsg3 RNA in various pooled tissues from healthy human donors. In comparison to the control skin sample, we could not detect any Dsg3 RNA in the bone marrow, spleen, or PBMCs **(Figure 2-1 A)**, despite the ability to detect other control RNAs (CD90, Stro-1) known to be expressed in these same samples **(Figure 2-1 B)**. Interestingly, we were able to detect the presence of Dsg3 RNA in the thymus, which does express Dsg3 under the *Aire* promoter[285] **(Figure 2-1 A)**.

In order to probe the Dsg3 autoAb repertoires from patients, we took a genetics-based approach wherein we isolated B cell clones from the circulating blood of PV patients with active disease to create antibody phage display (APD) libraries. These APD libraries are comprised of upwards of 10^8 randomly-paired light and heavy chains from a single patient[286], and allow us to conduct antigen-based enrichment to isolate clones of interest from the B cell repertoire. In addition, we are able to sequence these phage-expressing single chain variable fragments, or scFv, for genetic analyses. Lastly, by utilizing this approach, we are also equipped to produce monoclonal scFv for validation and functional studies.

We created IgG APD libraries from two patients with active disease **(Table 2-I, PV1 and PV2)**, and then subjected these libraries to Dsg3 ELISA-based panning to enrich for Dsg3-specific IgG clones. In addition to these two APD libraries, heterohybridoma panels were generated from two other patients with active disease **(Table 2-I, PV3 and PV4)** and clones were selected for Dsg3 specificity. A portion of mAbs isolated from patient 1 have been previously reported[282], but in total, we isolated 16 distinct heavy chains comprising six clonal lineages as defined by heavy chain CDR3. In patient 2, we isolated three heavy chain representing three clonal lineages. Patients 3 and 4 antibody libraries generated one unique antibody each; the mAb from patient 3

was previously reported as PVMAB786[287] . Of these clonal lineages, three bound to both Dsg 3 and Dsg1, while the remainder bound only to Dsg3, and was consistent across all unique heavy chain within each clonal lineage. Using a cutoff of 50% inhibition of Dsg3 ELISA-based binding in the presence of EDTA, most mAbs demonstrated a dependence on the presence of calcium to maintain Dsg3 binding **(Table 2-I)**. Immunoprecipitation of chimeric Dsg molecules shows that most mAbs bound the distal EC1 domain of Dsg3, with the exception of PVE4-8 from patient 1, as well as 3.2 and 4.2 from patient 2, which bound both the Dsg3 EC1 and EC3 domains. This domain recognition was recapitulated with serum from these same patients **(Table 2-II)**.

Interestingly, across twenty-two heavy chains comprising eleven clonal lineages, the only shared genetic characteristic common to these four patients' anti-Dsg3 repertoires was the utilization of the VH1-46 gene segment **(Table 2-I)**. These VH1-46 Abs were validated to bind Dsg3 by immunofluorescence (**Figure 2-2 A)**, and BIACORE analysis to quantitate the affinity of these mAbs **(Table 2-III)**. Based on epitope mapping **(Table 2-II)**, most bound the proximal EC1 domain which is thought to play a major role in Dsg3 *trans* interactions. In accordance with the Dsg3 epitopes bound by these VH1-46 autoAbs, all were able to induce an acantholytic blister upon injection into *ex vivo* human skin explants **(Figure 2-2 B)**. Sequence analysis of AK23[288, 289], a pathogenic Ab isolated from an active immune mouse model of PV, reveals that *VH1-46* is the closest human VH gene segment for this particular mAb based on amino acid sequence **(Figure 2-3)**, further highlighting the utilization of *VH1-46* in the autoimmune response to Dsg3 in both human and mouse.

Interestingly, when we conducted somatic mutation analyses on these VH1-46 Abs, they did not demonstrate high levels of somatic mutation in comparison to non-

VH1-46 mAbs (median 4 versus 8, P≤0.01 via Wilcoxon rank-sum test, **Table 2-IV**). Furthermore, the VH1-46 Abs did not demonstrate evidence of positive selection in the germinal center using a Bayesian estimation (BASELINe) test[290, 291] **(Table 2-IV)** due to a lower observed mutation frequency than expected under the null hypothesis of no selection. In contrast, one VH1-69 clonal lineage exhibited statistically significant evidence of positive antigen-driven selection, and another trended towards positive antigen-driven selection with several clones with a p-value from 0.05 to 0.10 **(Table 2-IV, light gray)**.

The low number of somatic mutations, coupled with the lack of positive selection based on mutational analyses, prompted us to determine whether these VH1-46 clones relied on somatic mutations to bind Dsg3, we reverted these clones to their germline (GL) sequence **(Table 2-V)** and expressed them as monoclonal scFv to test via ELISA and other *in vitro* assays for Dsg3 specificity. In case where there was more than one GL prediction due to D gene assignment, both predictions were made into monoclonal scFv. Dsg3 ELISAs showed that two VH1-46 GL Abs did not demonstrate Dsg3 reactivity **(Figure 2-4 A-B)**, but three other VH1-46 GL Abs maintained the ability to bind Dsg3 **(Figure 2-4 C-E)**. This is contrast to zero of five non-VH1-46 GL Abs **(Figure 2-4 F-J)** isolated from these same patients. Immunofluorescence (IF) experiments conducted on those three GL Abs that maintained Dsg3 reactivity correlated in terms of IF staining intensity with OD values by ELISA **(Figure 2-4 K)**. BIACORE analyses revealed that in concordance with a lower OD value by ELISA, 3.2 GL bound Dsg3 with eighty-fold lower affinity than its SM counterpart. On the other hand, PVE4-8 GL and 4.2 GL1/2 had comparable affinities (approximately two-fold lower) for Dsg3 as their SM derivatives **(Table 2-III)**. In addition, we tested these VH1-46 Abs for polyreactivity to nucleic acids

by both immunofluorescence and ELISA, and did not observe any reactivity to Hep2 cells by either methodology **(Figure 2-5)**.

We conducted mutagenesis experiments focusing on the two of five VH1-46 GL Abs that required somatic mutation for Dsg3 reactivity **(Figure 2-4 A-B)**. We observed that mutagenizing only two acidic residues in the CDRs to their polar analogs in the somatically-mutated F779 mAb abolished the ability of these mAbs to bind Dsg3 **(Figure 2-6 A-B)**. Conversely, insertion of these acidic residues into F779 GL1 and F706 GL2 partially rescued the ability of these GL Abs to bind Dsg3. Parallel mutagenesis experiments of the somatically-mutated F706 mAb and F706 GL could not be carried out due to inefficient scFv production. Paradoxically, F779 GL2 did not reproduce this phenotype **(Figure 2-6 A)**, suggesting that in addition to acidic residues, other mutations in the heavy chain CDR3 of F779 GL2 confer Dsg3 specificity. In addition, removal of acidic residues in the heavy chain CDR3 of PVE4-8 GL and 4.2 GL also abolished the ability of these Abs to bind Dsg3 **(Figure 2-6 C-D)**, further demonstrating that acidic amino acid residues are necessary and sufficient to confer Dsg3 reactivity in VH1-46 Abs.

In addition, to define the residues mediating GL reactivity in these VH1-46 mAbs, we carried out mutagenesis experiments focusing on the VH1-46 heavy chain CDR2. We mutagenized, in a step-wise fashion, the eight residues in the heavy chain CDR2, as well as the single residue in the -1 and +1 position relative to the heavy chain CDR to the VH1-02 residue in that same position **(Figure 2-7)**. ELISA showed that mutagenesis of only three residues in the VH1-46 CDR2 ameliorated binding to Dsg3 **(Figure 2-7, VH1-02 CDR2 only)**. Reactivity to Dsg3 was not dependent on the +1 position, as there was no alteration in Dsg3 affinity by ELISA **(Figure 2-7, PVE4-8 GL NYA)**. In contrast,

mutagenesis of the -1 position demonstrated a partial reduction in the ability to bind Dsg3 **(Figure 2-7, PVE4-8 GL RIN/WIN).**

We also determined whether inherent polymorphisms of the *VH1-46* locus could have contributed to acidic residues in the CDRs of these mAbs. There have been thirty-five single nucleotide variants (SNVs) identified in the *VH1-46* locus **(Table 2-VI)**. Sequencing of IgM cDNA from each patient revealed that all patients matched to the most common *VH1-46* allele, VH1-46*01. Of the thirty-five SNVs, eight do not alter the VH1-46 amino-acid sequence, nineteen SNVs encode a single amino-acid change in the FWRs, and eight encode a single amino-acid change in the CDR1 or 2. However, none of the thirty-five variants were observed in patients 1 and 2, while none of the eight CDR-relevant SNVs were observed in patients 3 and 4, and thus genetic polymorphisms did not contribute to acidic residues present in the CDRs of these patients.

One of the caveats of utilizing APD is the fact that the light and heavy chains from a patient are randomly paired together to form scFv. In order to determine the role for both the light and heavy chain in autoreactivity, we performed individual GL reversions of either the light or the heavy chain, and subjected these recombinant clones to ELISA to reactivity to Dsg3 and Dsg1. In all cases tested, reversion of the light chain was not sufficient to completely abolish binding, but did have some influence on the modulation of Dsg3 reactivity. However, reversion of heavy chain abolished Dsg3 reactivity in all but one case **(Figure 2-8)**, indicating that Dsg3 reactivity is predominantly encoded by the heavy chain, and not the light chain.

2.4 Discussion

Overall, these data uncover shared *VH1-46* gene usage in the anti-Dsg3 autoimmune response across four distinct individuals with active disease, suggesting a common developmental mechanism of autoimmunity. This likely occurs through an increased physiological fitness of GL VH1-46 Abs in regards Dsg3 specificity and the relatively low reliance on somatic mutation to bind to Dsg3, increasing their likelihood of contributing in the early autoimmune response to self.

The utilization of both phage display and heterohybridoma to clone the Ab repertoires of PV patients reduces the influence of both biases and artifacts resulting from the experimental technique. In support of the utilization of phage display, two separate studies, one using phage display and the other using single-cell PCR, identified the identical *VH5-51* heavy chain and light chain pairings in the autoAb response to transglutaminase in patients with celiac disease [292, 293]. The utilization of phage display as a technical approach likely results in the creation of nonphysiologic heavy light chain pairings. However, light chain shuffle experiments have resulted in generating the same pairings that were initially isolated through phage display[294]. While the role of the LC in conferring antigen specificity cannot be ignored completely, GL reversion experiments described above did highlight a predominant role for the heavy chain versus the light chain in conferring specificity **(Figure 2-8)**, further validating the use of phage display in studying the Ab response towards any antigen of interest.

The qPCR tissue studies **(Figure 2-1)** expand upon the theory of a lack of Dsg3 expression in the bone marrow and secondary lymphoid organs. In addition, the fact that we were able to detect Dsg3 mRNA in both the skin and the thymus, wherein Dsg3

expression varies dramatically, underscores the broad range of RNA concentration that this assay can detect. This suggests that under normal conditions, Dsg3 is not expressed in the tissue sites where both central and peripheral tolerance mechanisms take place, and thus B cells specific for this self-antigen would not be deleted from the repertoire. Importantly the detection of CD90 and Stro-1 in the bone marrow sample confirms the presence of stromal cells[295], which would be the population most likely to express Dsg3 in the bone marrow. However, as these tissue RNA samples were pooled from several different donors at a single timepoint, we can only make broad conclusions about the tissue-restricted expression of Dsg3 in the general population; perhaps in those expressing the PV HLA-susceptibility alleles there may be aberrant expression of Dsg3 in the periphery such that positive selection of Dsg3-binding B cells may be possible. Further experimental studies looking at this particular population may shed light on this idea. In addition, due to the nature of the experimental design, we cannot delineate between a large population of cells expressing a particular gene at low levels or vice versa. Lastly, it is not known whether Dsg3 may be trafficked to the secondary lymphoid organs via Langerhans cells upon tissue injury, as is the case for *Leishmania* antigens present in cutaneous tissue during infection[99]. However, based on these data describing the highly restricted expression of Dsg3, it is likely that the development of Dsg3-reactive B cell clones both in the bone marrow and the periphery occurs in the absence of tolerizing mechanisms. Thus, we wanted to determine at what stage Dsg3 autoreactivity arises in the B cell compartment which could arise as a byproduct of V(D)J recombination, somatic hypermutation, or both.

The only shared characteristic across the anti-Dsg3 Abs repertoires of these four patients was the presence of at least one mAb that utilized the VH1-46 gene segment.

This suggests that perhaps there may be something inherent to the VH1-46 gene sequence that might predispose Abs that utilize VH1-46 to be more physiologically fit to bind Dsg3 over other non-VH1-46 Abs, and thus may promote their detection early on in the autoimmune response. In support of an early role, these VH1-46s did not show evidence of positive selection **(Table 2-IV)**, suggesting a lower reliance of somatic mutation for antigen affinity. It should be noted that the BASELINe test is underpowered to detect positive selection in a sequence with low numbers of somatic mutation. This observation had not been described in an earlier report utilizing the heterohybridoma approach[283], however, none of the mAbs were validated to bind endogenous Dsg3 expressed in human skin, and thus may not possess any biological relevance. A second study[281] reported fifteen anti-Dsg3 mAbs, none of which utilized VH1-46, although the B cell enrichment strategy did not include plasma cells. In addition, one patient studied was under steroid therapy, which may also bias the observations within the autoAb repertoire of this patient. In contrast, the patients we studied were not on any systemic immunosuppressive therapy, and our selection strategies encompass all peripheral B cell subsets.

The fact that these VH1-46 mAbs largely replicate the Dsg3 epitope binding patterns of patient serum **(Table 2-II)** underscore the relevance of these Abs in contributing to the serum Ab specificity. Within PV1, we did not observe serum binding to EC3, despite isolating PVE4-8 which binds both EC1 and EC3, and may suggest that PVE4-8 does not comprise a large portion of the serum Abs. In regards to PV2, we did not observe a complete overlap of epitopes between patient serum and the mAbs we isolated, and may be due to the inability to sample the complete B cell repertoire from a peripheral blood sample.

All mAbs that we isolated recognized an epitope located within the EC1 domain, which is thought to mediate both *trans* and *cis* Dsg3:Dsg3 interactions[147, 148], and is likely responsible for the ability of these mAbs to induce a blister upon *ex vivo* human skin injection. Interestingly, three VH1-46 mAbs (PVE4-8, 3.2, and 4.2) also interacted strongly with EC3 of Dsg3. The nature of this interaction is unclear, as EC3 has not been described to mediate adhesion. It has been shown that combinations of "non-pathogenic" mAbs can induce a blister[168], potentially due to clustering of Dsg3 and/or activation of downstream signaling post-mAb binding[296, 297]. Whether this interaction with EC3 also contributes to pathogenicity in the context of PV is not known, but the relevance of these EC3-binding mAbs is still apparent as these are still interactions with a self-protein, and thus remain pathologic from the perspective of PV in that a vast majority of healthy individuals do not possess circulating anti-Dsg3 Abs.

In addition, the VH1-46 mAbs isolated can also induce a suprabasal blister upon *ex vivo* human skin injection **(Figure 2-2 B)**, highlighting their relevance in disease pathogenesis. Interestingly, restricted *VH1-46* gene usage has not been observed in the autoAb response to antigens relevant in other autoimmune blistering diseases, such as Dsg1 in pemphigus foliaceus[298, 299] , or the BP180[300] and BP230[300] antigens in bullous pemphigoid. Furthermore, a pathogenic murine mAb isolated from the active immune model uses murine VH1-53, which aligns most closely with *VH1-46* based on amino acid sequence **(Figure 2-3)**, and studies in paraneoplastic pemphigus, a related disease to PV, also isolated pathogenic VH1-46 mAbs[301], demonstrating that this *VH1-46* - observation in the Dsg3 Ab response can be seen across different contexts and even across different species.

Upon reversion of Dsg3-specific Abs to their unmutated GL sequence, we observed a varying degree of GL reactivity, wherein three of five VH1-46 Abs bound Dsg3 upon reversion, as opposed to zero of five non-VH1-46 Abs **(Figure 2-4)**. Looking at these three Dsg3-reactive GL Abs more closely, both GL revertants of 4.2 SM were strongly reactive, while 3.2 GL was lowly reactive by ELISA. Interestingly, this held true by immunofluorescence experiments, demonstrating that ELISA results correlate with intensity of binding by immunofluorescence as well as BIACORE analysis. Somatic mutation analyses in one GL-reactive VH1-46 clone demonstrated the specific dependence on amino acid residues in and around the heavy chain CDR2 **(Figure 2-7)**, as residues from the VH1-02 gene segment which is most closely related to VH1-46, could not confer reactivity to Dsg3. Overall, the binding of three VH1-46 Abs is independent of somatic mutation, suggesting that the inherently stochastic process of V(D)J recombination can confer Dsg3-reactivity to a subset of VH1-46 B cell clones exiting the bone marrow. This phenomenon appears to be specific to VH1-46 Abs, as none of the five non-VH1-46 mAbs we tested **(Figure 2-4 K)**, nor four others from a separate study[281], bound Dsg3 upon reversion. Taken together, these data further supports a germline-encoded fitness for certain antigens based on VH gene segment utilized to form a functional heavy chain.

Previous studies have described a large proportion of new emigrant B cell clones that demonstrate polyreactivity to nucleic acid, insulin, and lipopolysaccharide[46]. To ensure that these Dsg3-reactive GL Abs were not polyreactive, we tested these Abs for reactivity to nucleic acid by both immunofluorescence and ELISA, yet did not observe any reactivity to Hep2 cells **(Figure 2-5)**, suggesting that the self-reactivity of these clones is specific to Dsg3, and likely not a result of polyreactivity. Whether there are

other self-Ag that these VH1-46 clones may bind is not as clear, and further studies utilizing a large panel of self-Ag would elucidate this point.

Somatic mutation analyses reveal a lack of statistically-significant positive selection in the *VH1-46* mAbs tested, pointing away from a clonal lineage that acquired somatic mutations that would increase affinity for Dsg3 in the germinal center. In addition, mutagenesis experiments suggest that even in the context where somatic mutations are required for Dsg3 reactivity, only a low number of somatic mutations are necessary, as removal or insertion of only two replacement mutations in F779 and F706 were necessary and sufficient to confer Dsg3 binding **(Figure 2-6)**. This was apparent in Dsg3-reactive GL Abs as well, further supporting the role of acidic amino acids in mediating binding of mAbs to Dsg3, which can come from germline V(D)J sequence or somatic mutation.

In summary, these data suggests that a Dsg3-reactive B cell can arise from two developmental stages: V(D)J recombination in the bone marrow, or as a byproduct of somatic hypermutation in the periphery. Acidic amino residues, derived from either V(D)J recombination or somatic hypermutation, are necessary and sufficient to confer Dsg3 reactivity. Furthermore, the shared utilization of VH1-46 in the Dsg3 Ab repertoires across four patients is likely due to the requirement of zero to a low number of somatic mutations to become Dsg3-specific, and may increase these Abs probability of initiating an autoimmune response to Dsg3, ultimately leading to PV. This study suggests that the potential for generating Dsg-3 reactive B cell clones in the bone marrow is likely, and thus there should exist an appropriate method of tolerance to prevent these clones from differentiating into antibody secreting cells.

A lack of Dsg3-reactive T cells due to negative selection in the thymus may be essential in maintaining tolerance against Dsg3 in the broader population. However, in some cases, Dsg3-reactive T cells escape negative selection and enter circulation, as has been described in both healthy people and PV patients that express the HLA susceptibility alleles for PV[207, 302]. Perhaps during infection, a relaxation of negative selection occurs such that Dsg3-reactive B cells interact with antigen, acquire T cell help from a Dsg3-reactive T cell, and thus initiate an autoimmune response against Dsg3. Future studies to understand B cell tolerance checkpoints in the context of Dsg3, as well as potential triggers of the anti-Dsg3 autoimmune response will further our understanding of this disease and autoimmunity as a whole.

2.5 Figures

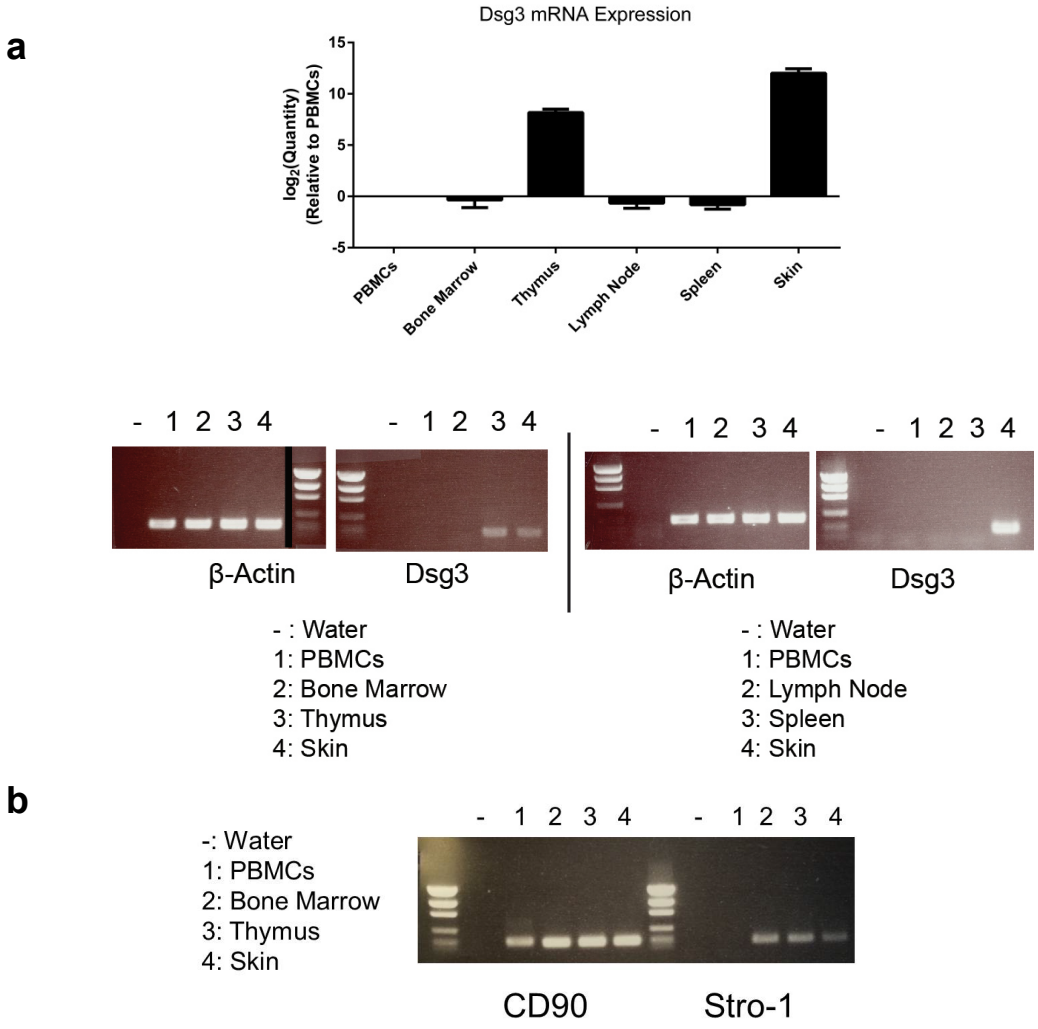


Figure 2-1. Desmoglein 3 is not detected in the bone marrow or peripheral lymphoid organs

Bulk RNA from human tissue samples was reverse-transcribed and subject to qPCR. Transcript quantities were quantitated after normalization to *β-actin*. A black line within a gel indicates splicing to include molecular weight marker within the same experiment. Data are representative of three independent experiments. Error bars indicate SEM.

Clone name(s)	Variable region gene usage			# unique clones	Dsg3	Dsg1	Calcium-sensitive
PV1	**V**	**D**	**J**				
PVE4-8	VH1-46 *01/03	DH3-22*01	JH6*02	1	+	-	+
(D3)1d/2c et al.	VH1-69*06	DH3-22*01	JH4*02	7	+	-	+
(D31)2/29	VH1-69 *06/09	DH6-19*01	JH4*02	1	+	+	+
(D3)3c/9 et al.	VH3-07*03	DH2-15*01	JH4*02	3	+	+/-	+
(D31)12b/6 et al.	VH4-04*02	DH5-12*01	JH2*01	3	+	+	-
(D3)4/30	VH3-30*04	D3-22*01	JH4*02	1	+	-	ND
PV2	**V**	**D**	**J**				
3.2	VH1-46 *01/03	DH5-12*01	JH4*02	1	+	-	+
4.2	VH1-46 *01/03	DH6-25*01/ DH3-22*01	JH6*02	1	+	-	+
VH5a	VH5-a*01	DH3-22*01	JH3*02	1	+	-	ND
PV3	**V**	**D**	**J**				
F706	VH1-46 *01/03 VK2-24*01	DH2-21*02 -	JH4*02 JL1*01	1	+	-	+
PV4	**V**	**D**	**J**				
F779	VH1-46 *01/03 VK2-24*01	DH6-19*01 -	JH4*02 JL2*01	1	+	-	+

Table 2-I. Characteristics of anti-Dsg3 monoclonal antibodies isolated from four PV patients

Unique clones within each clonal lineage are distinguished by distinct patterns of somatic mutation; in these lineages one representative clone is named with “et al.” to indicate the isolation of other mAbs with the same variable region gene usage and complementarity determining region (CDR) 3 but unique somatic mutations. ND, not determined. (D3) and (D31) Abs are listed as previously described[282].

	EC1	EC2	EC3	EC4	EC5
PV1 serum	X				
PVE4-8	X		X		
(D3)1d/2c	X*				
(D31)2/29	X				
(D3)3c/9	X				
(D31)12b/6	X				
PV2 serum	X	X	X	X	
3.2	X		X		
4.2	X		X		
VH5a	X				
PV3 serum	X				
F706 IgG4	X				
PV4 serum	X				
F779 IgG1	X				

*Dsg3 amino acids 1-161

Table 2-II. Epitope mapping of PV serum IgG and PV monoclonal antibodies

Chimeric constructs containing a single Dsg3 extracellular (EC) domain in a Dsg2 backbone were incubated with either serum or purified scFv and immunoprecipitated. The chimeric Dsg3-Dsg2 protein, if pulled down, was detected via Western blot. Data are representative of three biological replicates.

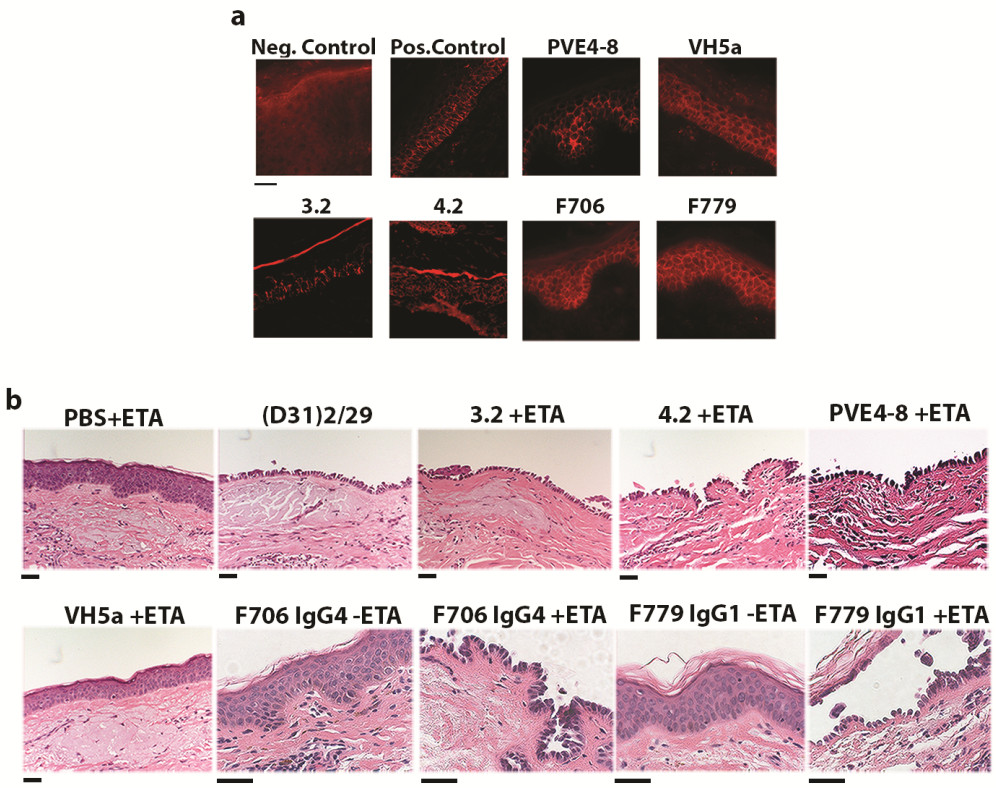


Figure 2-2. Anti-desmoglein 3 monoclonal antibodies induce an acantholytic phenotype

(a) Sections of normal human skin were incubated with monoclonal antibodies (mAb) expressed as single chain variable fragment (scFv). The negative control is an scFv mAb against an irrelevant antigen. The positive control is a previously characterized anti-Dsg3 mAb, (D31)2/29 [282]. Scale bar, 20 μM.

(b) *Ex vivo* human skin was injected with 50 μg scFv, subjected to a Nikolosky test, and incubated overnight. The next day, the tissue was embedded and sections were taken for histology. Scale bar, 100 μM.

Data are representative of three independent experiments.

scFv	ka ($M^{-1}s^{-1}$)	kd (s^{-1})	KD (M)	χ^2 value
PVE4-8 SM	5.15E+05	1.23E-03	2.39E-09	2.23
PVE4-8 GL	1.92E+06	2.36E-03	1.23E-09	5.83
3.2 SM	1.62E+06	2.31E-03	1.42E-09	1.45
3.2 GL	3.99E+04	7.03E-03	1.76E-07	1.43
4.2 SM	3.91E+06	7.12E-04	1.82E-10	1.1
4.2 GL1	2.40E+06	4.40E-04	1.83E-10	2.88
4.2 GL2	4.84E+05	3.75E-03	7.75E-09	1.2
F706 SM	9.50E+04	1.14E-03	1.20E-08	1.3
F706 GL1	No Binding			
F706 GL2	No Binding			
F779 SM	3.10E+05	5.48E-04	1.77E-09	1.11
F779 GL1	No Binding			
F779 GL2	No Binding			
(D3)1d/2c	9.00E+05	5.63E-05	6.30E-11	2.26
(D3)3c/9	8.30E+05	1.30E-04	1.60E-10	1.65
(D31)12b/6	6.70E+05	1.10E-04	1.60E-10	8.18
(D31)2/29	ka1 ($M^{-1}s^{-1}$)	kd1 (s^{-1})	KD1 (M)	χ^2 value
	2.10E+06	1.90E-02	9.00E-09	0.894
	ka2 ($M^{-1}s^{-1}$)	kd2 (s^{-1})	KD2 (M)	
	1.20E-02	2.00E-03	1.60E-01	

Table 2-III. Kinetic data of VH1-46 anti-Dsg3 monoclonal antibodies as measured by surface plasmon resonance.

Purified scFv were flowed over a CM5 biosensor chip coupled with recombinant Dsg3 protein. Background was adjusted using a reference cell to subtract nonspecific shifts in refractive index. All curves were initially fit to a 1:1 Langmuir binding model. For those mAbs with considerable bulk change or χ^2 values greater than 2, curves were also fitted to the heterogeneous ligand or conformational change models. Data represents 1-2 experiments over multiple concentrations.

Figure 2-3. The pathogenic mouse anti-Dsg3 mAb AK23 utilizes mouse VH1-53, which is the closest homolog of VH1-46 in the mouse

The human VH1-46 and murine VH1-53 germline amino acid sequences were aligned to the AK23 mAb sequence[288, 289].

A blue letter indicates a hydrophobic residue.

A black letter indicates a hydrophilic residue.

A red letter indicates a negatively charged residue.

A green letter indicates a positively charged residue.

The CDR1 and CDR2 sequences are boxed in blue and green, respectively. Asterisks indicate replacement mutations occurring in AK23 relative to the murine VH1-53 germline sequence; only one replacement mutation is observed in CDR2 of AK23.

ID	Observed Mutations				Total Mutations	Expected Mutation Frequencies				Expected Mutations				CDR		FWR	
	CDR		FWR			CDR		FWR		CDR		FWR		BASELINe		BASELINe	
	R	S	R	S		R	S	R	S	R	S	R	S	Sigma	P-value	Sigma	P-value
IGHV1-46*01/03 Clonal Lineage 1																	
3.2	2	4	4	1	11	0.170	0.052	0.584	0.194	0.00	0.00	0.00	0.00	-0.465	-0.281	-1.070	-0.046
IGHV1-46*01/03 Clonal Lineage 2																	
4.2	4	2	9	1	16	0.170	0.052	0.584	0.194	3.84	1.20	13.27	4.69	0.629	0.190	0.152	0.420
IGHV1-46*01/03 Clonal Lineage 3																	
F706	5	2	5	8	20	0.16	0.049	0.586	0.206	0.00	0.00	0.00	0.00	-0.189	-0.367	-1.490	-0.002
IGHV1-46*01/03 Clonal Lineage 4																	
F779	4	0	7	5	16	0.158	0.048	0.584	0.21	4.34	1.35	15.00	5.30	0.282	0.325	-0.503	-0.183
IGHV1-46*01/03 Clonal Lineage 5																	
PVE4-8	4	2	6	3	15	0.170	0.052	0.584	0.194	3.67	1.14	12.69	4.49	0.158	0.397	-0.695	-0.116
IGHV1-69*06 Clonal Lineage 1																	
(D31)2/29	10	1	8	4	23	0.167	0.052	0.577	0.204	3.84	1.20	13.27	4.69	1.080	0.017	-0.369	-0.245
IGHV1-69*06 Clonal Lineage 2																	
(D3)1i/4d	9	1	9	7	26	0.167	0.052	0.577	0.204	4.34	1.35	15.00	5.30	0.540	0.125	-0.699	-0.070
(D3)1d/2c	8	1	9	4	22	0.167	0.052	0.577	0.204	3.67	1.14	12.69	4.49	0.870	0.054	-0.258	-0.307
(D3)1g/2e	9	1	8	7	25	0.167	0.052	0.577	0.204	4.18	1.30	14.43	5.10	0.540	0.125	-0.811	-0.048
(D3)1b/3a	8	1	8	5	22	0.167	0.052	0.577	0.204	3.67	1.14	12.69	4.49	0.700	0.088	-0.540	-0.149
(D3)1c/2a	9	1	8	5	23	0.167	0.052	0.577	0.204	3.84	1.20	13.27	4.69	0.811	0.053	-0.540	-0.149
(D3)1e/2d	8	1	6	6	21	0.167	0.052	0.577	0.204	3.51	1.09	12.12	4.28	0.554	0.133	-0.956	-0.037
(D3)1a/4a	9	1	8	5	23	0.167	0.052	0.577	0.204	3.84	1.20	13.27	4.69	0.811	0.053	-0.540	-0.149
IGHV3-7*03 Clonal Lineage																	
(D3)3c/9	5	2	8	7	22	0.178	0.048	0.563	0.21	3.92	1.06	12.39	4.62	-0.183	-0.373	-0.892	-0.030
(D3)3a/9	5	2	7	6	20	0.178	0.048	0.563	0.21	3.56	0.96	11.26	4.20	-0.072	-0.455	-0.907	-0.035
(D3)3b/8	5	2	6	5	18	0.178	0.048	0.563	0.21	3.20	0.86	10.13	3.78	0.055	0.455	-0.925	-0.042
IGHV4-4*02 Clonal Lineage																	
(D31)12b/6	3	1	9	4	17	0.194	0.045	0.542	0.219	3.30	0.77	9.21	3.72	-0.165	-0.413	-0.168	-0.368
(D31)12a/5	5	0	11	4	20	0.194	0.045	0.542	0.219	3.88	0.90	10.84	4.38	0.512	0.211	0.228	0.352
(D31)12c/7	5	0	9	3	17	0.194	0.045	0.542	0.219	3.30	0.77	9.21	3.72	0.775	0.128	0.297	0.329
IGHV5-a*01 Clonal Lineage																	
VH5a	9	1	18	9	37	0.178	0.047	0.593	0.181	6.59	1.74	21.94	6.70	0.148	0.369	-0.386	-0.158
IGHV3-30*04 Clonal Lineage																	
(D3)4/30	2	0	2	4	8	0.194	0.044	0.556	0.206	1.55	0.35	4.45	1.65	-0.373	-0.329	-1.430	-0.034

Table 2-IV. BASELINe test for antigen-driven selection in Dsg3-specific mAbs

BASELINe sigma value quantitates the strength of negative or positive selection based on somatic mutation patterns and allows one to compare strength of selection across every Ab tested[290, 291]. Negative and positive selection are indicated by – and + symbols before the p values, respectively. While two VH1-46 mAbs exhibit significant evidence of negative selection against replacement mutations in the FWRs ($p<0.05$, highlighted in dark gray), no VH1-46 mAb demonstrates statistically significant evidence of positive antigen-driven selection in the CDRs. Several non-VH1-46 clonal lineages demonstrate evidence of negative selection in the FWRs that is statistically significant. In terms of positive selection, VH1-69 clonal lineage 1 shows statistically significant evidence of positive antigen-driven selection in the CDRs, while VH1-69 clonal lineage 2 CDRs trends towards significance ($0.05<p<0.1$, highlighted in light gray).

Table 2-V. CDR sequences of anti-Dsg3 mAbs

mAbs that are somatically mutated are indicated with an SM. Those that are germline-reverted are indicated with a GL, and reverted germline residues are underlined. Point-mutations are indicated with a blue, underlined residue.

Clone	CDR1 LC	CDR2 LC	CDR3 LC	VL gene	JL gene	CDR1 HC	CDR2 HC	CDR3 HC	VH gene	DH gene	JH gene
PVE4-8 SM	SSDVGGYNY	EVN	SSYAGSNNLV	IGLV2-8*01	IGLJ2*01	GYTFTAYY	INPSGGIA	ARDRQGFDLDV	IGHV1-46*01	IGHD3-22*01	IGHJ6*02
PVE4-8 GL	SSDVGGYNY	EVS	SSYAGSNNLV			GYTFTSYY	INPSGGSI	ARDRQGFDLDV			
PVE4-8 GL HC D→N	SSDVGGYNY	EVS	SSYAGSNNLV			GYTFTSYY	INPSGGST	ARNRQGFNLNV			
3.2 SM	SSDIGRYNF	EIY	SSYVGNNDLV			GYTFTSYY	INPSGGIA	ARDLGGFDFDY			
3.2 GL	SSDVGGYNY	EVS	SSYVGNNDLV	IGLV2-8*01	IGLJ7*01	GYTFTSYY	INPSGGSI	ARDLGGFDFDY	IGHV1-46*01	IGHD5-12*01	IGHJ4*02
4.2 SM	SSDVGGYNY	EVS	SSYAGSNNAV			GYIFTSHY	INPSGGKT	ARDQSLGMDV			
4.2 GL1	SSDVGGYNY	EVS	SSYTSSNNAV	IGLV2-14*01	IGLJ3*02	GYIFTSYY	INPSGGST	ARDQRLGMDV	IGHV1-46*01	IGHD6-25*01	IGHJ6*02
4.2 GL1 HC D→N	SSDVGGYNY	EVS	SSYTSSNNAV			GYIFTSYY	INPSGGST	ARNQRLGMNV			
4.2 GL2	SSDVGGYNY	EVS	SSYTSSNNAV	IGLV2-14*01	IGLJ3*02	GYIFTSYY	INPSGGST	ARDHSLGMDV	IGHV1-46*01	IGHD3-22*01	IGHJ6*02
4.2 GL2 HC D→N	SSDVGGYNY	EVS	SSYTSSNNAV			GYIFTSYY	INPSGGST	ARNHSLGMNV			
4.2 SM LC D/E → N/Q	SSNVGGYNY	QVS	SSYAGSNNAV			GYIFTSHY	INPSGGKT	ARDQSLGMDV			
4.2 SM HC D → Q	SSDVGGYNY	EVS	SSYAGSNNAV			GYIFTSHY	INPSGGKT	ARNQSLGMNV			
F706 SM	ETLVHSDGNTY	KIS	TQSTDFPWT	IGKV2-24*01	IGLJ1*01	GYTFTSYY	IDSRGST	ARGVGTLDH	IGHV1-46*01	IGHD2-21*02	IGHJ4*02
F706 GL2	QSLVHSDGNTY	KIS	TQSTDFPWT			GYTFTSYY	INPSGGST	ARGVGTLDH			
F706 GL2 + D/E	ESLVHSDGNTY	KIS	TQSTDFPWT			GYTFTSYY	IDPSGGST	ARGVGTLDH			
F706 GL	QSLVHSDGNTY	KIS	MQATQFPWT			GYTFTSYY	INPSGGST	ARVVVTLDY			
F779 SM	ETLVHSDGNTY	KIS	MQATEFPYT	IGKV2-24*01	IGLJ2*01	GNTFTTYS	IDPSGGST	ARSIESISGRTLGY	IGHV1-46*01	IGHD6-19*01	IGHJ4*02
F779 GL1	QSLVHSDGNTY	KIS	MQATEFPYT			GYTFTSYY	INPSGGST	ARSIESISGRTLGY			
F779 GL1 + D/E	ESLVHSDGNTY	KIS	MQATEFPYT			GYTFTSYY	IDPSGGST	ARSIESISGRTLGY			
F779 SM D/E → N/Q	QTLVHSDGNTY	KIS	MQATEFPYT			GNTFTTYS	INPSGGST	ARSIESISGRTLGY			
F779 GL2	QSLVHSDGNTY	KIS	MQATQFPYT			GYTFTSYY	INPSGGST	ARSIEYSSGWTLGY			
F779 GL2 + D/E	ESLVHSDGNTY	KIS	MQATEFPYT			GYTFTSYY	IDPSGGST	ARSIEYSSGWTLGY			
(D3)1d/2c SM	SSNIAGNT	YND	ATWDEDVNGWV			GGTFDKYA	IIPMLGAP	ARDKAAYYESGYYYIGDF			
(D3)1d/2c GL	SSNIGSNT	SNN	AAWDDSLNGWV	IGLV1-44*01	IGLJ3*02	GGTFSSYA	IIPIFGTA	ARDKAAYYESGYYYFDY	IGHV1-69*06	IGHD3-22*01	IGHJ4*02
(D31)2/29 SM	KLGDKY	QDR	QAWDSSTAV			GGTFGNYA	IIPTLDLL	ARGGDYSGWYNFDY			
(D31)2/29 GL	KLGDKY	QDS	QAWDSSTAV	IGLV3-1*01	IGLJ3*02	GGTFSSYA	IIPILGIA	ARGGDYSGWYNFDY	IGHV1-69*06/09	IGHD6-19*01	IGHJ4*02
(D3)3c/9 SM	SSYVGIFNL	EGD	YSYVAGSDLWV			GLPFNSYW	INQDGNEK	ASGGVVDFDH			
(D3)3c/9 GL	SSDVGSYNL	EGS	CSYAGSSTLWV	IGLV2-23*01/03	IGLJ3*02	GFTFSSYW	IKQDGSEK	ARDGVDYFDY	IGHV3-07*03	IGHD2-15*01	IGHJ4*02
(D31)12b/6 SM	SSHIGSNY	SND	AAWDDGQGGV			GGSISSNHW	IYHNGST	ARGHHRTGFRGYPSHWYFDL			
(D31)12b/6 LC SM/HC GL	SSHIGSNY	SND	AAWDDGQGGV			GGSISSSNW	IYHSGST	AREWHRTGYSGYPSYWYFDL	IGHV4-04*02	IGHD5-12*01	IGHJ2*01
VH5a SM	SSNIRNNY	DDN	GTWDSSQSVGV			GPNFSNYW	IDPFDGYT	ARINYYDSGHHSDADYM			
VH5a LC GL/HC SM	SSNIGNNY	DNN	GTWDSSLSAGV	IGLV1-51*01	IGLJ3*02	GPNFSNYW	IDPFDGYT	ARINYYDSGHHSDADYM			
VH5a LC SM/HC GL	SSNIRNNY	DDN	GTWDSSQSVGV			GYSFTSYW	IDPSDSYT	ARINYYDSGGYYSDAFDI	IGHV5a*01	IGHD3-22*01	IGHJ3*02

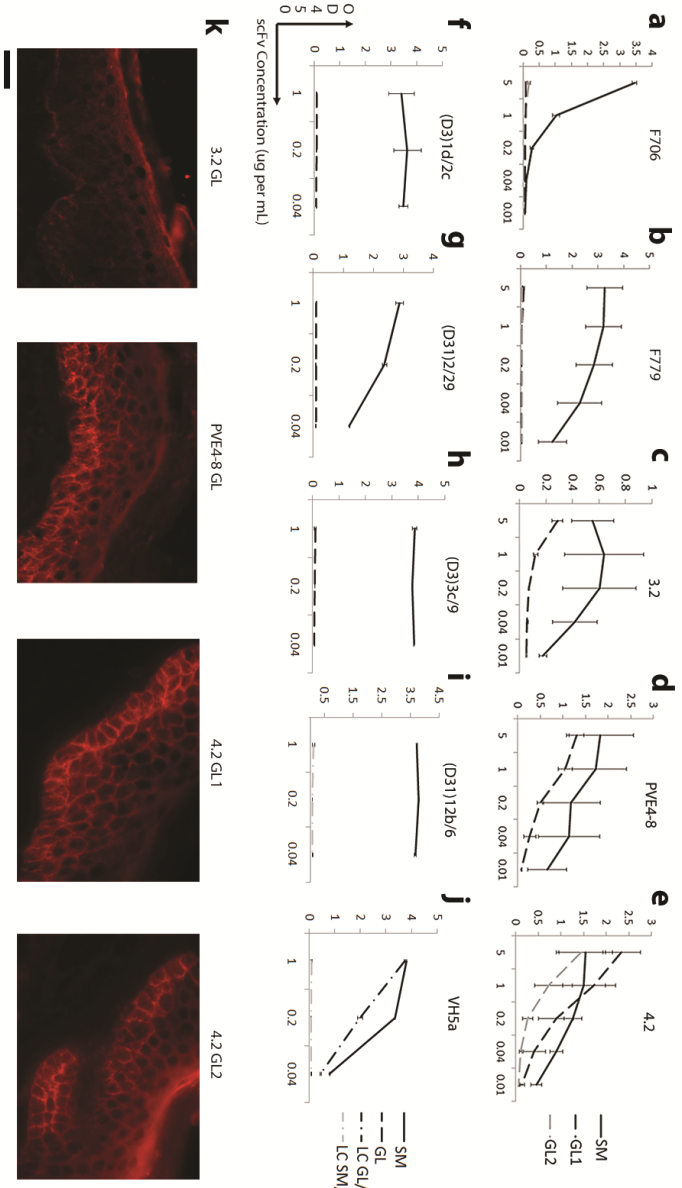


Figure 2-4. VH1-46 mAbs require few to no somatic mutations to bind Dsg3

(a-j) Purified scFv was tested for the ability to bind Dsg3 by ELISA at various concentrations. Black bar indicates somatically-mutated (SM). Black dashed lines indicates germline version 1 (GL1). Gray dashed lines indicates germline version 2 (GL2). Black long dash dot lines indicate a construct with a germline light chain and a somatically mutated heavy chain (LC GL/HC SM). Gray long dash dot lines indicate a construct with a somatically mutated light chain and a germline heavy chain (LC SM/HC GL).

(k) Native human skin tissue was incubated with purified scFv and staining was detected by immunofluorescence. Scale bar, 20 μM.

Error bars indicate SEM. Data are representative of three to five independent replicates.

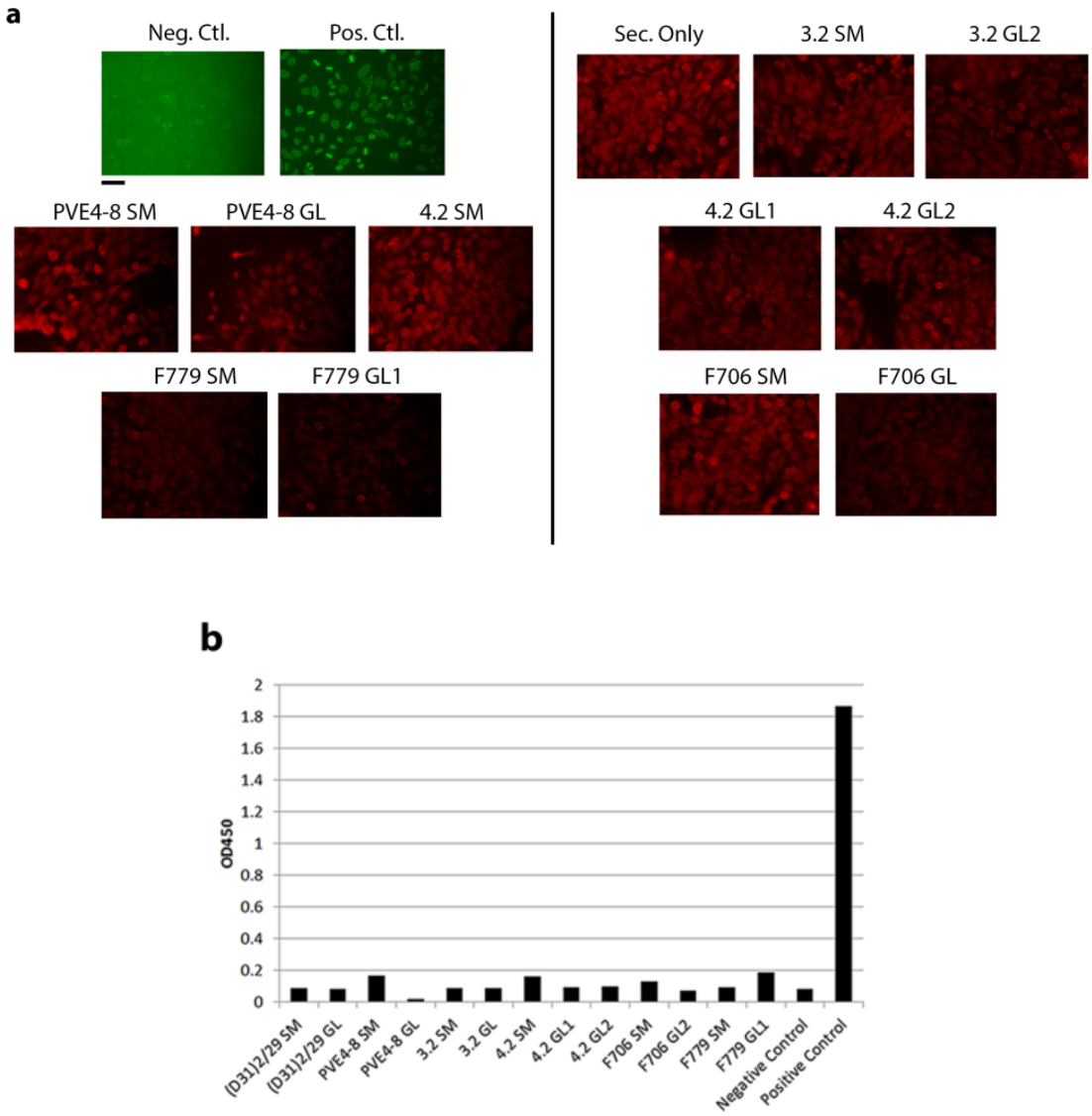


Figure 2-5. Pemphigus vulgaris mAbs do not demonstrate Hep 2 polyreactivity

Patient mAbs were tested for reactivity to Hep2 cells by both immunofluorescence **(a)** and ELISA **(b)**. Commercial negative (Neg. Ctl.) and positive (Pos. Ctl.) controls are displayed. Secondary only control (Sec. Only) indicates staining of cells with anti-hemagglutinin, horseradish peroxidase-conjugated antibody alone, which was used to detect scFv. Scale bar, 20 μM. Data are representative of 1-2 experiments tested at multiple concentrations.

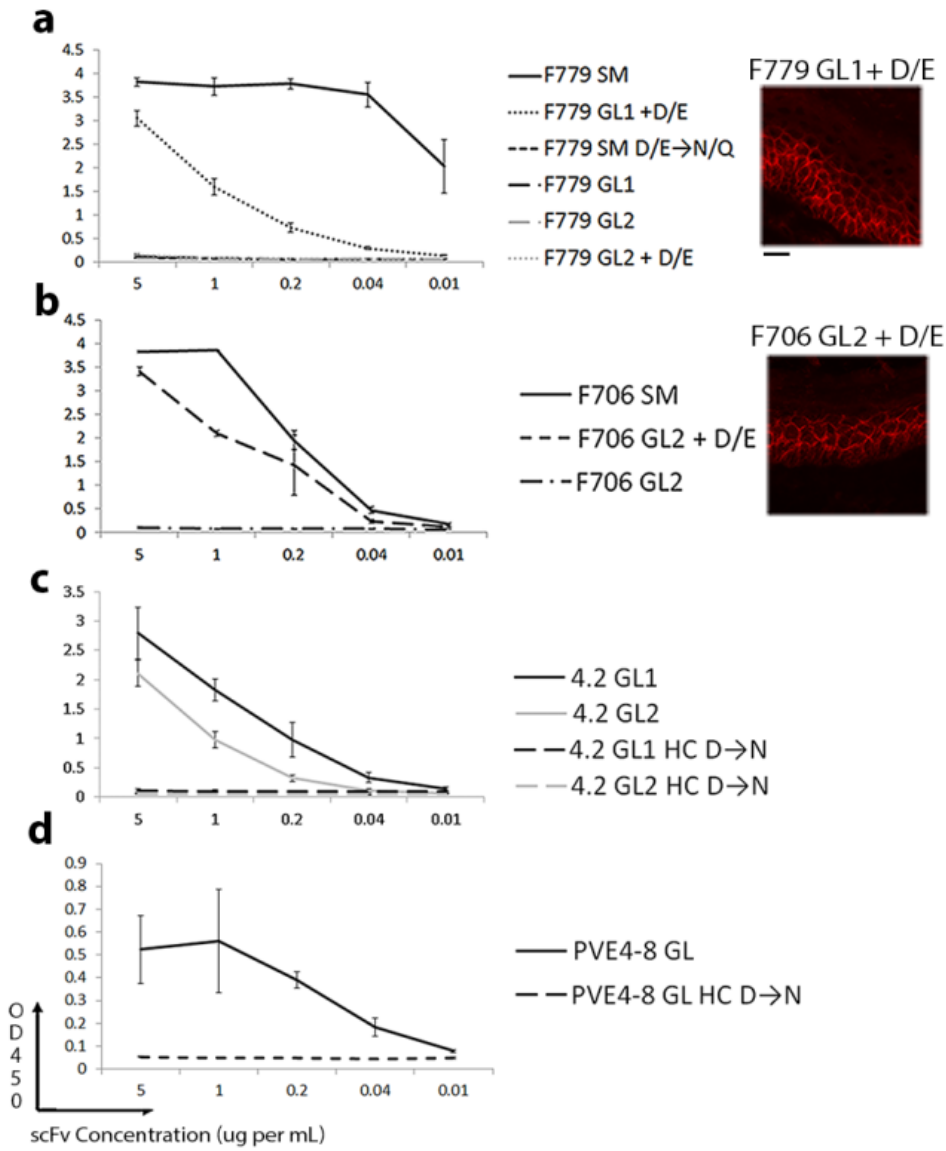
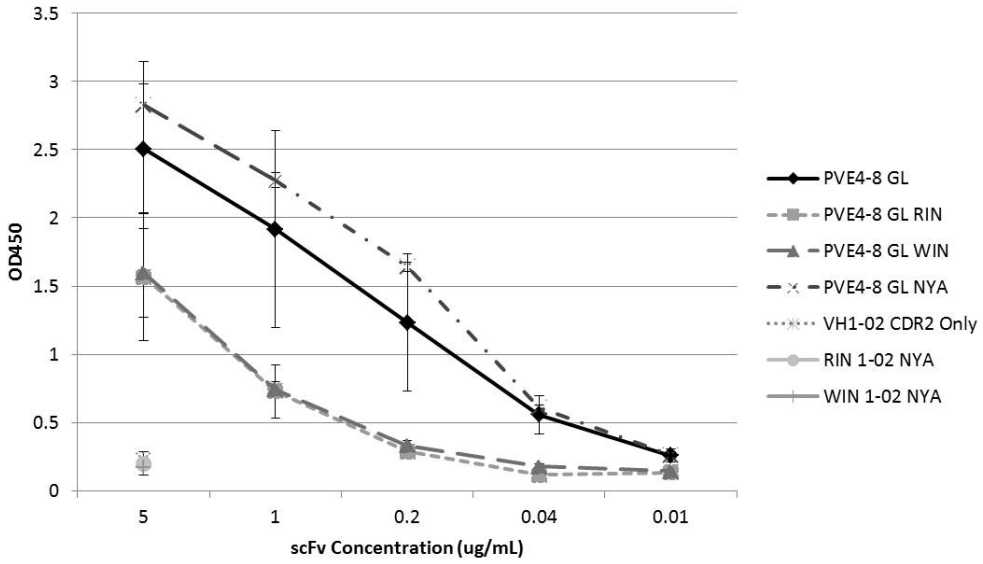


Figure 2-6. Acidic amino acid residues in the CDRs confer Dsg3 binding

(a-d) Selected acidic amino residues were mutated to their respective germline (GL) constructs, or mutated to their polar analogs in both somatically-mutated (SM) or GL constructs. Point mutants were expressed as scFv, purified, and tested for Dsg3 specificity by ELISA and immunofluorescence where relevant. Scale bar, 20 μM. Error bars indicate SEM. Data are representative of three independent experiments.



Construct ID	Amino Acid		
	Flank	CDR2	Flank
VH1-46 GL	LEWMGI	INPSGGST	SYAQKFQ
VH1-46 RIN	LEWMGR	INPSGGST	SYAQKFQ
VH1-46 WIN	LEWMGW	INPSGGST	SYAQKFQ
VH1-46 NYA	LEWMGI	INPSGGST	NYAQKFQ
VH1-46 1-02 CDR2	LEWMGI	INPNSGGT	SYAQKFQ
VH1-46 RIN 1-02 NYA	LEWMGR	INPNSGGT	NYAQKFQ
VH1-46 WIN 1-02 NYA	LEWMGW	INPNSGGT	NYAQKFQ

Figure 2-7. Dependence on heavy chain CDR2 residues for VH1-46 germline reactivity to Dsg3

Residues (black, underlined) in PVE4-8 GL were mutated to the corresponding residue in either VH1-02*01/VH1-02*05 or VH1-02*02/VH1-02*03/VH1-02*04 (blue, underlined). Point mutants were expressed as scFV and subjected to Dsg3 ELISA over multiple concentrations. Error bars indicate SEM. Data are representative of two independent replicates.

SNV reference	minor allele frequency	majority codon	variant	silent	non-silent	AA change
rs374571144	-	GTG	GTT	x		
rs370958214	-	CAG	CAA	x		
rs184383009	0.001	CTG	TTG	x		
rs191958250	-	GTG	ATG		x	V->M
rs375773401	-	GGG	GCG		x	G->A
rs61732934	-	GCT	GGT/GTT		x	A->G/A->V
rs188566927	0.001	AAG	CAG		x	K->Q
rs370633142	-	AAG	AAC		x	K->N
rs377180003	-	GGG	GGA	x		
rs372749973	-	GCC	ACC		x	A->T
rs61747196	0.002	GTT	ATT		x	V->I
rs61995748	0.002	AAG	ATG		x	K->M
rs192285778	-	GCA	TCA		x	A->S
rs187613260	0.0005	TAC	GAC		x	Y->D
rs377014204	-	TAC	TAT	x		
rs374579537	-	AGC	AGG		x	S->R
rs182132309	0.001	TAC	ATC		x	Y->I
rs369870124	-	TAT	TTT		x	Y->F
rs376685264	-	ATG	GTG		x	M->V
rs144704015	0.002	ATG	ATA		x	M->I
rs367562305	-	CCT	TCT		x	P->S
rs376171941	-	GAG	GAA	x		
rs371609422	-	CCT	CTT		x	P->L
rs190309173	-	GGT	AGT		x	G->S
rs185595166	0.002	AGC	ACC		x	S->T
rs181189514	-	ACA	GCA		x	T->G
rs368402773	-	TAC	AAC		x	Y->N
rs149338091	0.001	AAG	AGG		x	K->R
rs55801711	0.008	TTC	TTG		x	F->L
rs371133633	-	GGC	GAC		x	G->D
rs368616898	-	ACC	ATC		x	T->I
rs147211698	0.003	ACG	ATG		x	T->M
rs370428883	-	ACG	ACA	x		
rs376062317	-	GTG	ATG		x	V->M
rs11848941	0.156	GCG	GCA/GCT	x		

Table 2-VI. Single nucleotide variants (SNVs) of the VH1-46 gene in the human genome

35 previously reported VH1-46 SNVs in the 1000 genome project (http://www.ncbi.nlm.nih.gov/variation/tools/1000genomes) are listed along with their respective observed minor allele frequency. As a comparison, the predominant codon sequence, the variant sequence, and whether the resulting nucleotide variant is silent or non-silent, are also listed. CDR1 and CDR2 residues are boxed in blue and green, respectively. None of the CDR1 or CDR2 SNVs were found in the four PV patients studied.

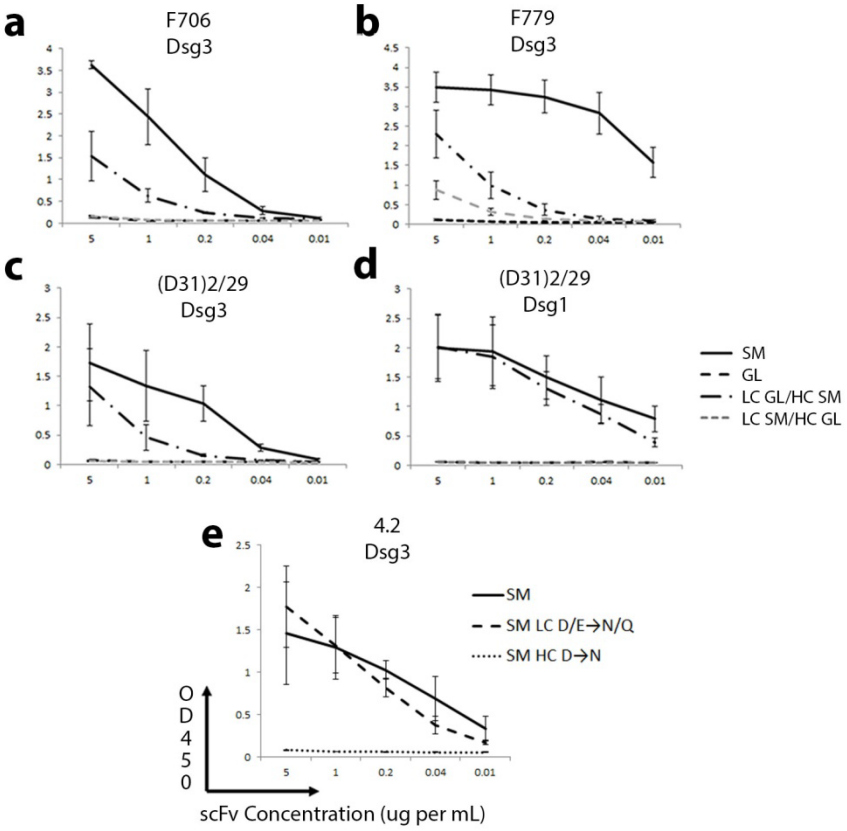


Figure 2-8. Determinants of Dsg3 autoreactivity are predominantly encoded within the heavy chain

(a-d) Light and heavy chains were individually reverted while maintaining the somatic mutations in the heavy and light chain (LC GL/HC SM, LC SM/HC GL respectively). Chimeric constructs were expressed as scFv and tested for Dsg3-binding by ELISA. Somatically mutated (SM) constructs are indicated with a solid black line.SM indicates somatically mutated. GL indicates germline reverted.

(e) Acidic residues in either the light chain CDR1 and CDR2 or the heavy chain CDR3 were mutated to their polar analogs (SM LC D/E→N/Q and SM HC D→N, respectively), expressed as scFv, and subjected to Dsg3 ELISA.

Error bars indicate SEM across three independent experiments.

CHAPTER 3: DETERMINANTS OF VH1-46 ANTIBODY CROSS-REACTIVITY TO DESMOGLEIN 3 AND ROTAVIRUS VP6

3.1 Abstract

Evidence of shared VH1-46 gene usage in an antibody response to an antigen has only been primarily described in pemphigus vulgaris (PV) and in the immune response to the rotavirus VP6 antigen. This interesting connection between an autoimmune response to a self-antigen and an immune response to a foreign antigen prompted us to determine whether rotavirus may be a potential trigger for the development of PV and explain the tolerance of autoreactive clones. To evaluate whether desmoglein (Dsg) 3/VP6 cross-reactive heavy chains occur in humans, we produced combinatorial BCR phage display libraries from two PV patients with active disease, a PV patient in remission, and a normal individual. Across these four libraries, we identified six cross-reactive heavy chains in the repertoire of the PV patient in remission, and notably all cross-reactive heavy chains utilized VH1-46. Functional testing identified two of these cross-reactive VH1-46 heavy chains that both disrupt keratinocyte adhesion as well as inhibit rotavirus infection, indicating the potential for VH1-46 heavy chains to have both pathologic autoimmune and protective immune functions. In addition, we performed mutational analyses to determine what structural features are required for a VH1-46 BCR to react to these two antigens. Taken together, these studies suggest that certain VH1-46 B cell populations may be predisposed to Dsg3 and VP6 cross-reactivity, but multiple mechanisms prevent the onset of autoimmunity after rotavirus exposure.

3.2 Introduction

To combat the numerous foreign insults a typical human would encounter on a daily basis, there must be a diverse B cell repertoire of a similar magnitude to prevent infection. The heterogeneity of the human antibody (Ab) repertoire, which can be upwards of 10^{11} specificities per individual[28, 29], arises from two distinct, largely stochastic processes of B cell receptor (BCR) diversification: V(D)J recombination and somatic hypermutation.

Given these immunological processes to vary the BCR repertoire, within the context of an immune response to an antigen, certain BCRs will have an inherent advantage over others simply due to its nucleotide sequence. Thus, any biases in representation would suggest advantageous characteristics of an optimal BCR for that particular antigen. These characteristics could be a specific amino acid motif, a certain heavy chain-light chain pairing, or an overrepresentation of a singular VH gene segment. A prominent example is in the human antibody response to influenza, wherein a phenylalanine within the heavy chain CDR2 that mediates interaction with hemagglutinin likely explains the overrepresentation of VH1-69 in several individuals[303-305].

VH bias in an immune response to an antigen has been described not just in response to foreign antigen, but in the context of self-antigen as well. Dsg3 is a desomosomal cadherin responsible for mediating adhesion in stratified squamous epithelia. It is the autoantigen targeted in pemphigus vulgaris (PV), a blistering disease characterized by acantholysis, which a separation of the spinous layers of the epidermis and can occur both in the skin and the oral cavity[143, 213]. Passive transfer of purified anti-Dsg3 immunoglobulin (Ig) from PV patients is necessary and sufficient to cause an acantholytic phenotype in several disease models [213-215]. Furthermore, this phenotype is

independent of both complement and the constant region of the Ab[217, 306], underscoring the role for specific autoAb binding to Dsg3 in mediating disease.

We have previously characterized the autoAb repertoires of four PV patients with active disease and discovered across these unrelated patients a common utilization of VH1-46 in their anti-Dsg3 repertoires, which is likely due to the reliance on zero to few somatic mutations to bind Dsg3 located predominantly in the heavy chain CDR2[167]. Interestingly, VH1-46 gene usage has also been observed in the immune response to the rotavirus VP6 protein[266, 274], which has also been observed to exhibit binding in the absence of high levels of somatic mutation[267, 274]. In addition, two anti-VP6 VH1-46 mAbs depend on somatic mutations in and around the heavy chain CDR2[279], similarly to the anti-Dsg3 VH1-46 mAbs we have described in PV. Therefore, we wanted to determine whether rotavirus may act as an immunological trigger in the autoimmune response to Dsg3 by permitting cross-reactive heavy chains to exist in the repertoire.

Directed ELISA testing of previously isolated VH1-46 IgG mAbs from PV patients reveal that one of five cross-react to VP6. Cross-screening three combinatorial IgM antibody phage display (APD) libraries from PV patients reveal VH1-46 heavy chains that cross-react to VP6 in one patient. Furthermore, two of these VH1-46 heavy chains are able to both inhibit rotavirus replication and keratinocyte adhesion *in vitro*, suggesting that Dsg3-reactive VH1-46 heavy chains may persist in the repertoire due to the ability to cross-react to VP6 in some individuals, indicating the potential for VH1-46 Abs to have both pathologic autoimmune and protective immune functions.

3.3 Results

First, we utilized ELISA to determine whether previously isolated VH1-46 single chain variable fragments (scFv) from four patients with active disease[167]. In addition, two VH1-46 mAbs (RV6-25, RV6-26) isolated from a rotavirus-exposed individual[307] were cloned and expressed as scFv. One of five somatically mutated (SM) PV-derived VH1-46 heavy chain demonstrated cross-reactivity to VP6 (4.2), while neither RV6-25 nor RV6-26 demonstrated cross-reactivity to Dsg3 **(Figure 3-1 A)**. These clones were then reverted to their germline (GL) VDJ sequence and expressed as scFv. PVE4-8 GL and 4.2 GL1 exhibited borderline negative levels of cross-reactivity upon reversion, as the lower end of the standard deviation range fell below the cutoff value for VP6 reactivity. In addition, neither RV6-25 nor RV6-26 demonstrated cross-reactivity to Dsg3 upon reversion, but maintained reactivity to VP6 **(Figure 3-1 B)**.

To determine whether we could isolate cross-reactive APD clones that bound both Dsg3 and VP6, we utilized two screening techniques: single and double antigen selection. The former technique was conducted as published[286], and allowed us to examine the anti-Dsg3 or anti-VP6 repertoires independently. The latter technique, wherein we alternated between Dsg3- and VP6-based selection using recombinant human Dsg3 and rotavirus double-layered particles (DLPs) containing surface VP6 in its native state, enriches for the opportunity of isolating cross-reactive APD clones. These approaches were applied to several patient libraries at several stages of disease[308] **(Table 3-I)**.

To expand upon the focused testing of previously isolated clones, we conducted a more thorough examination of the IgG repertoire via the above methods. A majority of the Dsg3-based selection data has been previously published[167, 282, 308], and largely is

enriched in VH1-46 utilization. As expected due to Dsg3 ELISA values below the cutoff value for a diagnosis of PV **(Table 3-I)**, the library that was isolated from a PV patient in remission did not generate Dsg3-reactive clones (data not shown).

In contrast, VP6-based selection revealed strong utilization of the VH4 family in the IgG repertoires of these patients. We did not observe a predominance of VH1-46 gene usage in any of the IgG APD libraries we screened by either VP6-based or double antigen selection. Interestingly, in one patient (PV3) where we were able to sample from distinct stages of disease, a new clone emerged that was not isolated in the previous timepoint **(Table 3-II)**. These clones were expressed as scFv and validated to bind VP6 but not Dsg3 by ELISA **(Figure 3-2).** Double antigen selection did not reveal any cross-reactive clones in the single library we subjected to cross-panning (data not shown).

Given the rarity of cross-reactive clones in the IgG compartment, as well as the role for heavy chain CDR2 mutations in the VH1-46 response to both Dsg3 and VP6, we conducted mutagenesis experiments to characterize the heavy chain CDR2 somatic mutation patterns that could lead to cross-reactivity. Somatic mutations that were found in either two VP6-reactive VH1-46 mAbs, RV6-25 and RV6-26, were mutagenized into various VH1-46 backbones that represented three different conditions **(Figure 3-3)**:

1.) A SM VH1-46 mAb that demonstrated cross-reactivity (4.2 SM)
2.) A GL VH1-46 that did not demonstrate cross-reactivity (F779 GL2)
3.) A GL VH1-46 mAb that demonstrated marginal cross-reactivity (PVE 4-8 GL)

In total, we tested six permutations: RV6-25 or RV6-26 mutations in three distinct VH1-46 backbones. Mutagenesis was carried out in a step-wise fashion, so to characterize

each region (-1 flank, CDR2, +1 flank) and its role in cross-reactivity within each backbone. These clones were then expressed as scFv and subjected to Dsg3 and VP6 ELISAs.

In the first condition (4.2 SM), mutagenesis of two amino acids in the heavy chain CDR2 to the respective RV6-25 residues led to a reduction in binding to Dsg3. Interestingly, these two residues abolished the ability of 4.2 SM to bind to VP6. Subsequent mutagenesis of the -1 and +1 positions relative to the heavy chain CDR2 resulted in a complete loss of binding to Dsg3 without any alteration of VP6 reactivity **(Figure 3-4 A).** Mutagenesis of 4.2 SM with RV6-26 somatic mutations resulted in a similar loss of binding to VP6, but did not markedly affect Dsg3 reactivity **(Figure 3-4 B)**. Insertion of RV6-25 or RV6-26 somatic mutations into F779 GL2 did not increase its affinity for either Dsg3 or VP6 above cutoff OD values **(Figure 3-4 C,D)**.

Similar to the first condition, when RV6-25 somatic mutations were inserted into PVE4-8GL, the ability of this mutant to bind Dsg3 was completely lost, without a concomitant increase in VP6 reactivity **(Figure 3-4 E)**. Insertion of a single RV6-26 somatic mutation within the CDR2 into PVE4-8 GL did not largely affect reactivity to either antigen **(Figure 3-4 F, purple line)**. However, when the -1 residue, along with the single residue in the CDR2 were mutated to their respective RV6-26 residues, there was an increase in reactivity to both Dsg3 and VP6 **(Figure 3-4 F, orange line)**. Upon subsequent mutagenesis of the +1 residue to the relevant RV6-26 residue, reactivity to VP6 was abolished without a marked change in Dsg3 reactivity **(Figure 3-4 F, pink line)**. A summary of the mutagenesis studies is shown in **Table 3-III**.

Given that unmutated IgM B cell receptors (BCRs) are formed in the bone marrow and have not undergone antigen exposure, Dsg3/VP6 cross-reactive potential might occur in all individuals and not just those with PV. To enrich for a starting population that is inherently more polyreactive than the IgG compartment[46], we took a more comprehensive approach and screened combinatorial IgM APD libraries from two patients with active disease (PV8 and PV16), one patient in remission (PV1c), and a healthy individual (CH) **(Table 3-I)**. Dsg3-based selection revealed VH1-46 usage in all PV patient libraries tested. VH1-46 was not over-represented in the initial libraries tested; we observed only two VH1-46 heavy chains in a total of 104 clones analyzed across the four libraries **(Table 3-IV)**. The anti-Dsg3 IgM repertoire from PV1c, the patient in remission, was heavily VH1-46-enriched, while PV8 and PV16, which are derived from patients in active disease, demonstrated much broader VH gene usage. Interestingly, the healthy individual demonstrated an anti-Dsg3 IgM repertoire that was enriched in VH3-23, and did not demonstrate any VH1-46 gene usage **(Tables 3 V-VIII)**. IgM clones were then expressed as scFv and validated to bind various antigens by ELISA **(Figure 3-5)**.

Double-antigen enrichment of these IgM APD libraries revealed VH1-46 clones in one (PV1c) of four libraries tested. VH1-46 was not enriched in the APD IgM library isolated from PV8, PV16, nor the healthy individual; in fact, the VH families utilized in these libraries were relatively diverse compared to PV1c. The healthy individual demonstrated the least amount of diversity from a much lower number of heavy chains compared to the other libraries tested **(Tables 3 V-VIII)**. Five clones isolated from PV16 during double-antigen enrichment were also isolated during Dsg3-based selection **(Table 3-VII, gray)**. Four clones isolated from double-antigen enrichment in PV8, PV16,

and CH demonstrated polyreactivity to Dsg3, VP6, BSA, and/or Hep2 antigens by ELISA **(Figure 3-5)**. However, immunofluorescence studies with these polyreactive clones did not demonstrate surface staining characteristic of a Dsg3-reactive mAb in human skin **(Figure 3-6)**.

In contrast to the other libraries, PV1c was VH1-46 predominant upon double-antigen selection; we isolated seven distinct VH1-46 heavy chains from this patient. Interestingly, two of the seven VH1-46 clones from the cross-screen were also found during Dsg3-based selection **(Table 3-V, gray)**. ELISA and immunofluorescence revealed that six of the nine VH1-46 clones from PV1c bound to Dsg3 and VP6, two VH1-46 clones bound only to Dsg3, and a single VH1-46 clone bound solely to VP6 **(Figure 3-7)**. While PV1c IgM DVDV-8 did not appear to stain the surface of keratinocytes in human skin **(Figure 3-7 B)**, immunofluorescence on monkey esophagus, a more sensitive substrate than human skin[309, 310], revealed distinct staining characteristic of Dsg3 specificity **(Figure 3-7 C)**.

The evident cross-reactivity of these VH1-46 clones prompted us to determine whether this binding to VP6 would possess any level of antiviral function. To test this, we utilized a previously established *in vitro* assay that exploits the ability of some mAbs to neutralize DLPs within a host cell via lipid-mediated introduction of DLPs pre-incubated with scFv[311]. Of the seven VH1-46 tested in this assay, two demonstrated a marked reduction in the number of rotavirus foci compared to the control antibody at 25 ug/mL **(Figure 3-8 A)**. This reduction in foci by the VH1-46 scFv derived from PV1c was comparable to RV6-26, which has been previously shown to inhibit rotavirus replication[245, 279]. Titration of scFv revealed that PV1c VH1-46 IgM DVDV-7 and -8 were

able to inhibit replication at a concentration of 25 ug/mL and 6.25 ug/mL, respectively **(Figure 3-8 B)**.

Given these two VH1-46 heavy chains' relevance in protection against rotavirus, we wanted to determine whether they could also play a role in acantholytic blister formation. Upon incubation with keratinocytes *in vitro*, PV1c IgM DVDV-7 and -8 inhibited Dsg3-mediated adhesion at varying levels corresponding to their respective Dsg3 affinities based on ELISA **(Figure 3-9)**, suggesting that these two cross-reactive VH1-46 clones demonstrate the ability to both inhibit rotavirus infection and promote keratinocyte dissociation within the skin.

3.4 Discussion

In total, these data suggests that a VH1-46 heavy chain can exhibit cross-reactivity to Dsg3 and VP6, but this is largely modulated by sequences in both the CDR2 and CDR3 of the heavy chain. Evidence of cross-reactive Abs contributing to autoimmunity have been described by several groups in a myriad of different contexts. Certain anti-*Streptococcus* antibodies can interact with cardiac myosin[312] along with other human proteins [313], and likely contribute to the symptoms of rheumatic heart disease. In regards to other bacterial triggers in autoimmunity, there has been a strong association of previous infection with *C. jejuni* and the development of Guillain-Barre syndrome in humans[314]. Interestingly, experiments in rabbits immunized with *C. jejuni* have demonstrated a much more rapid development of limb weakness than controls[315]. In fogo sevalgem, an endemic form of pemphigus, antibodies that cross-react with Dsg1 and sand fly antigens[316] have also been shown to protect against leishmaniasis[317], a

disease that is also prevalent in the regions affected by fogo selvalgem. It is intriguing that in these studies, the induction of autoimmunity does not appear to be 100% penetrant; the *C. jejuni* study in rabbits, for example, resulted in only four of the ten animals demonstrating limb weakness[318]. A similar observation of <100% penetrance was also reported in the context of a rat model of rheumatic heart disease[319].

A challenge in delineating the connection of rotavirus and PV is the lack of shared linear epitopes between Dsg3 and VP6. Because of the apparent lack of molecular mimicry, we came across this potential connection due to the shared VH gene usage across the two antibody responses. We term this association the "shared VH gene usage theory". The utilization of certain VH genes in an antibody response may increase physiological fitness in the context of binding one antigen, but not others. Based on this concept, we posit that the "shared VH gene usage theory" states that due to a shared VH gene usage between an immune response to a foreign antigen and an autoimmune response to a self-antigen, there must be some cross-reactive Abs that overlap between these two responses at some point in time. This has also been described for VH4-34 9G4 anti-idiotype antibodies in systemic lupus erythematosus which bind sugar moieties on erythrocytes[50, 51] and arise after acute infection with *Mycoplasma pneumoniae* and/or Epstein-Barr virus[320, 321]. However, Abs of these type seem to bind a plethora of different self-antigens in addition to the I/I sugar moieties, including ssDNA, dsDNA, apoptotic cells[322], and could be considered polyreactive in comparison to the VH1-46 Abs we described which are cross-reactive only to VP6 and Dsg3. In addition, it is not clear whether these anti-idiotype antibodies are protective against *Mycoplasma pneumoniae* and/or Epstein-Barr virus, and contrasts what we

observe in two cross-reactive VH1-46 antibodies that demonstrate both pathologic and protective functions in PV and rotavirus, respectively.

To test this theory, we conducted directed testing of previously isolated VH1-46 IgG clones, and observed that only one of five VH1-46 heavy chains derived from PV patients exhibited cross-reactivity to VP6 **(Figure 3-1 A),** suggesting a low level of cross-reactivity. GL reversion experiments further underscored a low amount of cross-reactivity in these clones **(Figure 3-1 B)**. This was not entirely unexpected, as these clones were isolated using single-antigen selection, and thus may have favored high affinity clones solely targeting Dsg3.

To probe the IgG repertoire more extensively for cross-reactive clones, we subjected IgG APD libraries to both single and double antigen selection. VP6-based selection of the IgG APD libraries tested did not reveal strong utilization of the VH1-46 in the anti-VP6 IgG repertoire. This is not surprising, as the predominance of VH1-46 is markedly lower in the $IgD^{-}CD27^{+}$ B cell subset compared to the naïve $IgD^{+}CD27^{-}$ subset[278], and is likely due to the acquisition of somatic mutations in non-VH1-46 clones that increase affinity such that the VH1-46 predominance is diminished over time. However, we were unable to isolate VH1-46 clones from the VP6-based selection. This may be due to these clones being outcompeted by the extremely high affinity clones we did recover, which dominated the repertoire by the final round of selection **(Table 3-II)**. Furthermore, results from the double antigen selection of an IgG library did not reveal any cross-reactive clones, suggesting that cross-reactivity in the IgG compartment is rare. Future studies potentially conducing deep sequencing analyses of the anti-Dsg3 and anti-VP6 Ab repertoires may reveal shared lineages that would identify a common clonal ancestor that would otherwise be lost during double-antigen selection.

Somatic mutation analyses of the heavy chain CDR2 region suggest that the threshold to acquire cross-reactivity through the process of somatic mutation varies between VH1-46 mAbs. Cross-reactivity was observed in one permutation, but the majority of the mutagenesis experiments resulted in a loss of antigen reactivity **(Figure 3-4, Table 3-III)**. It is likely that there is a role of the light chain and/or CDR1/3 of the heavy chain in conferring cross-reactivity, but these remain to be tested. A previous report in rotavirus depicting the interactions between the VH1-46 mAb RV6-26 and VP6 have shown that RV6-26 relies on positively-charged regions located in the heavy chain CDR2 to bind to a negatively-charged patch of residues on the VP6 surface[245]. In contrast, the VH1-46 response in PV seems to depend on negatively-charged acidic residues, both in the heavy chain CDR2 and elsewhere, for Dsg3 specificity which can arise through either V(D)J recombination or somatic mutation[167]. This stark difference in VH1-46 mutation characteristics between a VP6 and Dsg3 response may explain the dearth of cross-reactive VH1-46 clones, as it would be unlikely for a single VH1-46 clone to acquire somatic mutations that could increase affinity to both antigens concurrently.

Given the nature of affinity maturation in these two antibody responses, perhaps by probing the antigen-experienced IgG compartment, there is a loss of cross-reactivity in favor of high affinity for a single antigen. We then generated IgM APD libraries from these same patients as well as a healthy individual in order to be able to probe the IgM heavy chain repertoire.

Three IgM APD libraries, two from PV patients and one from a healthy individual, did not reveal any IgM heavy chains that were specific for both Dsg3 and VP6 **(Tables 3VI-VIII)**, and may point to the rarity of creating a cross-reactive clone through V(D)J recombination. We did isolate clones that demonstrated polyreactivity that utilized a

variety of VH3 family members as well as VH1-69 **(Figure 3-5 E)**. Isolation of these clones is likely a result of panning the IgM repertoire, which has been shown to be inherently polyreactive[46]. IF studies reveal that the heavy chains do not bind Dsg3 on the surface of keratinocytes **(Figure 3-6)**, and thus it is unclear of these heavy chains' role in the development of PV, but the existence of these clones may point to an alternative etiology of autoreactive clones originating from a polyreactive ancestor in a subset of individuals.

The fact that we did not isolate cross-reactive clones specific for Dsg3 and VP6 from three of these IgM libraries suggests that the process of generating APD libraries does not create artificial cross-reactive clones. In addition, the lack of cross-reactive clones in these three APD individuals may allude to a viral trigger that is distinct from rotavirus and has yet to be discovered. High-throughput polyclonal Ab studies utilizing panels of human viral antigens may elucidate additional viral triggers of PV outside of rotavirus.

It is intriguing that we did isolate a Dsg3-reactive heavy chain from a healthy individual, given that this individual does not have PV **(Table 3-I)**. This further supports that V(D)J recombination is certain to produce autoreactive heavy chains. It is then the burden of tolerance to inhibit these autoreactive clones from proliferating, differentiating, and ultimately contributing to standing serum antibody titers and a diagnosis of PV.

In contrast, we isolated nine VH1-46 heavy chains from a PV patient in remission, six of which were validated to be cross-reactive **(Figure 3-7)**. This VH1-46 enrichment is not an artifact of phage display, as we detected only two VH1-46 clones out of ninety from the three unpanned APD libraries we tested **(Table 3-IV)**. VH1-46

utilization has also been described as representing only ~2% of the circulating repertoire[323], and thus to recover six validated VH1-46 clones suggests strong enrichment of VH1-46 within the cross-reactive Ab response of this individual.

Out of these six cross-reactive VH1-46 clones, two demonstrated the ability to inhibit rotavirus replication within a host cell **(Figure 3-8)**. This ability to hamper the rotavirus life cycle points to a functional role for these VH1-46 cross-reactive heavy chains during an active rotavirus infection. Perhaps this clarifies why these heavy chains can persist in the repertoire despite being autoreactive, and in certain individuals, these may eventually mature into high affinity anti-Dsg3 antibody secreting cells that can cause PV. Furthermore, these two VH1-46 clones can also cause keratinocyte dissociation **(Figure 3-9)**, underscoring their role in causing PV should they be expressed as soluble antibody.

It is intriguing that we also isolated four cross-reactive VH1-46 heavy chains that do not inhibit rotavirus replication, because it is known that not all anti-VP6 Abs inhibit replication[246-248]. These cross-reactive VH1-46 heavy chains may not necessarily be protective, but their mere existence does suggest that the “shared VH gene usage theory”, at least within the context of rotavirus and PV, is theoretically possible, since we did observce Ab cross-reactivity to VP6 and Dsg3 to some extent.

What differentiates the one IgM APD library wherein we isolated cross-reactive clones from the other two IgM APD PV patient libraries is the stage of disease; PV1c was taken from a patient in remission after rituximab, while the other libraries were from two patients with active disease. While short-term alterations in VH gene distribution have been observed in a two rheumatoid arthritis patients after treatment with rituximab,

one of these patients, while demonstrating a normal VH gene distribution before treatment, exhibited statistically significant modulations of VH gene distribution in both directions across certain VH gene families[324]. However, these same alterations in VH gene distribution were not observed in a larger, thirteen person cohort of patients with anti–myelin-associated glycoprotein neuropathy post-rituximab treatment[325]. Regardless, in the context of PV1, the ablation of the B cell compartment with rituximab may have promoted the overrepresentation of cross-reactive clones in the new B cell repertoire, perhaps in an effort to repopulate the B cell niche. Longitudinal studies of these patients' B cell repertoires both before and after treatment would elucidate whether disease stage and/or rituximab plays any role(s) in the detection of cross-reactive heavy chains.

The observation that a majority of VH1-46 IgM heavy chains demonstrate cross-reactivity suggests that that inherently stochastic process of V(D)J recombination produces autoreactive clones. While this conclusion itself is not that surprising, the fact that more people do not have PV given these observations is interesting, and suggest that there are robust tolerance mechanisms in place prevent these clones from differentiating into antibody-secreting cells. Given the rarity of PV, as well as the distinct pattern of somatic mutations in the Dsg3 and VP6 IgG response, it is possible that these cross-reactive clones are initially activated based on reactivity toward a foreign antigen, but likely do not develop IgG reactivity toward Dsg3, as a large majority of the population do not demonstrate circulating Dsg3-reactive T cells[302] to provide help to these cross-reactive clones in becoming autoreactive ASCs.

While VH1-46-based crossreactivity to Dsg3 and VP6 is rare in the class-switched IgG compartment, our data demonstrate that there is a theoretical possibility that a class-switched VH1-46 B cell clone could become cross-reactive via somatic

hypermutation. In addition, unmutated VH1-46 heavy chains demonstrate preponderance towards being cross-reactive to VP6 and Dsg3; in some cases, even being able to both inhibit rotavirus replication and promote the dissociation of keratinocytes. The immune system is then tasked with inhibiting these cross-reactive clones from persisting and/or differentiating into antibody-secreting cells. In a large majority of the population, this is likely the case given the high prevalence of rotavirus infection. However, further studies need to be done to better understand why in the few people that do develop PV, what developmental event(s) occurred that led to this loss in tolerance.

3.5 Figures

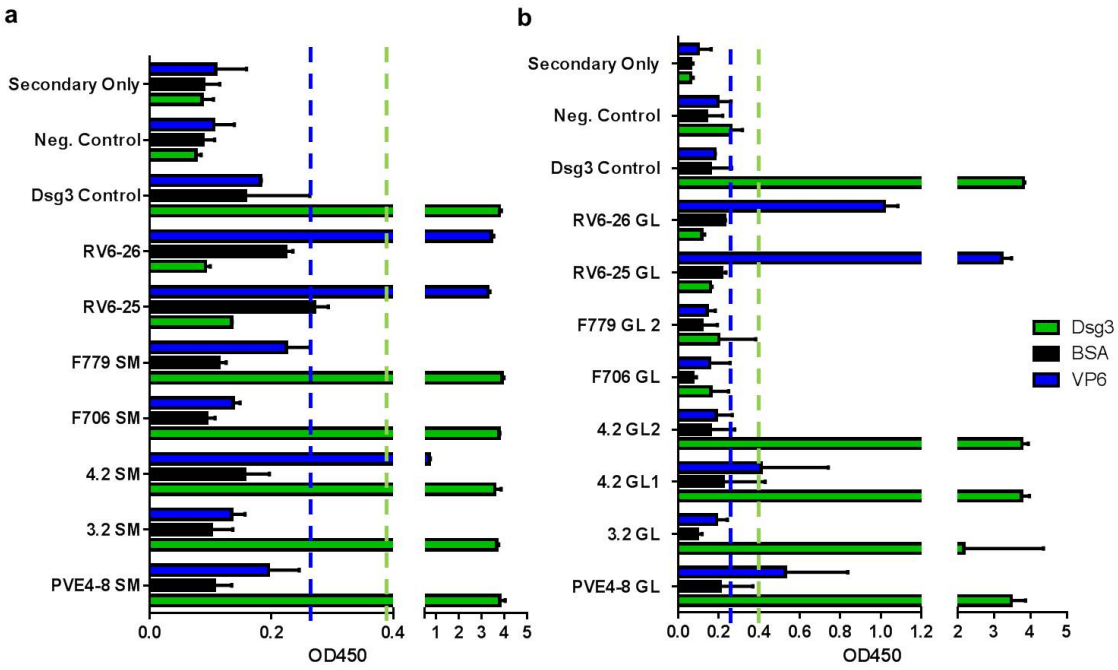


Figure 3-1. Cross-reactivity to VP6 is rare in previously isolated IgG Abs

(a) Previously isolated, somatically mutated (SM) IgG Abs from patients with active PV[167, 282, 287] or a rotavirus-exposed individual[307] were tested for cross-reactivity to VP6 by ELISA.

(b) These same Abs were reverted to germline (GL) and subjected to ELISA.

Green bars indicate Dsg3 reactivity. Black bars indicate bovine serum albumin reactivity. Blue bars indicate VP6 reactivity. Blue dashed line indicates cutoff value of 0.263 for VP6 reactivity. Green dashed line indicates cutoff value of 0.398 for Dsg3 reactivity. Dsg3 control is clone (D31)2/29. Error bars indicate SEM. Data are representative of two independent experiments.

Patient	Libraries Produced	Disease Stage	Dsg3 ELISA INDEX	Symptoms
PV1	IgG	Active	115	Mucocutaneous
PV1c	IgM, IgG	Remission	5	None
PV3	IgG	Active	70	Mucocutaneous
PV3a	IgG	Relapse	150	Mucocutaneous
PV8	IgM	Active	151	Mucocutaneous
PV16	IgM	Active	140	Mucocutaneous
CH	IgM	Healthy	ND	None

Table 3-I. Characteristics of patients studied

Standard cutoff value for a positive diagnosis based on Dsg3 ELISA is 20 as per manufacturer's indication. ND indicates not determined. PV3 in this study is listed as PV2 in Chapter 2 to maintain consistency with a collaborator's recent manuscript[308].

a

							ELISA Reactivity		
Clone	GL VH gene	GL DH gene	GL JH gene	CDR1	CDR2	CDR3	Dsg3	VP6	BSA
PV1 IgG V4-2	IGHV4-34*01	IGHD6-19*01	IGHJ5*02	GGPFNIDQ	INHAGGT	ARGGRAVPDAGENWFDS	-	+	-
PV1c IgG V4-9	IGHV1-2*02	IGHD5-12*01	IGHJ4*02	GYTFTGYY	INPSSGGT	ARAKDTWRPLSAYDL	-	+	-
PV1c IgG V4-14	IGHV4-4*02	IGHD6-19*01	IGHJ6*02	GGSISSSDW	IHHTGST	ARARLSVGYGMDV	-	+	-
PV3 IgG V4-1	IGHV4-39*01	IGHD7-27*01	IGHJ2*01	SISSSSYF	VFYTGESN	ARRPQLGIVSWYFDL	-	+	-
PV3a IgG V3-1	IGHV4-39*01	IGHD3-22*01	IGHJ3*02	GGSVSSTNYY	LFNSGKS	ARHSFTDYNDAFDI	-	+	-

b

						ELISA Reactivity		
Clone	GL VL gene	GL JL gene	CDR1	CDR2	CDR3	Dsg3	VP6	BSA
PV1 IgG V4-2	IGKV4-1*01	IGKJ3*01	QSVLYSSNNKNY	WAS	QQYFSPPFT	-	+	-
PV1c IgG V4-9	IGKV4-1*01	IGLJ3*01	QSVLYSSNNKNY	WAS	HQYYSTPPT	-	+	-
PV1c IgG V4-14	IGLV3-1*01	IGLJ3*01	KLGDKY	QDS	QAWDSSRVV	-	+	-
PV3 IgG V4-1	IGKV3-20*01	IGKJ1*01	QSVSSSY	GAS	QQYGRSPWT	-	+	-
PV3a IgG V3-1	IGLV1-47*01	IGLJ3*02	SSNIGSNY	RNN	ATWDDSLSGWV	-	+	-

Table 3-II. IgG clones isolated from VP6-based selection

APD libraries were subjected to four rounds of enrichment against VP6. Germline (GL) assignments are displayed, along with CDR sequences. V3/4 indicates isolation from rounds 3 or 4 of VP6-based selection, respectively. Reactivity to various antigens was also tested by ELISA.

(a) Heavy chains
(b) Light chains

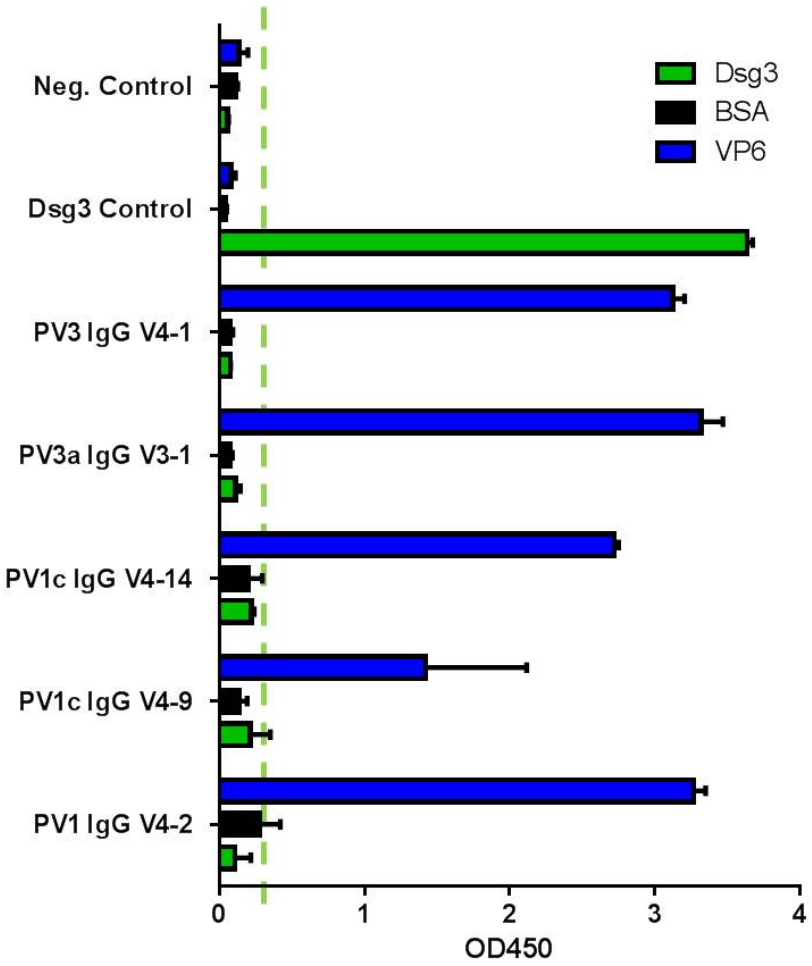
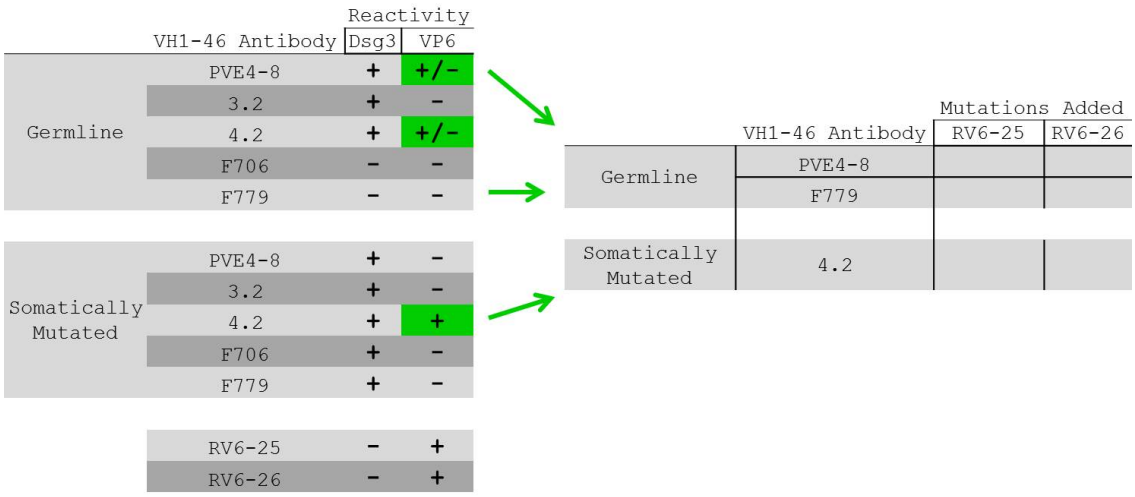


Figure 3-2. IgG clones isolated by VP6-based selection are mono-specific for VP6

IgG clones isolated from APD were tested for ELISA reactivity against Dsg3 (green), BSA (black), and VP6 (blue). Green dashed line indicates cutoff value of 0.225 for Dsg3 reactivity. Dsg3 control is clone (D31)2/29. Neg. Control is a mAb against an irrelevant antigen. Error bars indicate SEM. Data are representative of two independent experiments.

a



b

Construct ID	Amino Acid Flank	CDR2	Flank
VH1-46 GL	LEWMGI	INPSGGST	SYAQKFQ
VH1-46 6-25 CDR2	LEWMGI	INPKGGYT	SYAQKFQ
VH1-46 GV 6-25 CDR2	LEWmGV	INPKGGYT	SYAQKFQ
VH1-46 GV 6-25 CDR2 TYAE	LEWmGV	INPKGGYT	TYAEKFQ

Construct ID	Amino Acid Flank	CDR2	Flank
VH1-46	LEWMGI	INPSGGST	SYAQKFQ
4.2 SM	LEWMGI	INPSGGKT	TFAQKFQ
4.2 SM RV6-25 CDR2	LEWMGI	INPKGGYT	TFAQKFQ
4.2 SM GV RV6-25 CDR2	LEWMGV	INPKGGYT	TFAQKFQ
4.2 SM GV 6-25 CDR2 TYAE	LEWMGV	INPKGGYT	TYAEKFQ

Construct ID	Amino Acid Flank	CDR2	Flank
VH1-46 GL	LEWMGI	INPSGGST	SYAQKFQ
VH1-46 6-26 CDR2	LEWMGI	INPSDGST	SYAQKFQ
VH1-46 GM 6-26 CDR2	LEWMGM	INPSDGST	SYAQKFQ
VH1-46 GM 6-26 CDR2 YYAQR	LEWMGM	INPSDGST	YYAQRFQ

Construct ID	Amino Acid Flank	CDR2	Flank
VH1-46	LEWMGI	INPSGGST	SYAQKFQ
4.2 SM	LEWMGI	INPSGGKT	TFAQKFQ
4.2 SM RV6-26 CDR2	LEWMGI	INPSDGST	TFAQKFQ
4.2 SM GM RV6-26 CDR2	LEWMGM	INPSDGST	TFAQKFQ
4.2 SM RV6-26 CDR2 YYAQ	LEWMGI	INPSDGST	YYAQRFQ
4.2 SM GM RV6-26 CDR2 YYAQ	LEWMGM	INPSDGST	YYAQRFQ

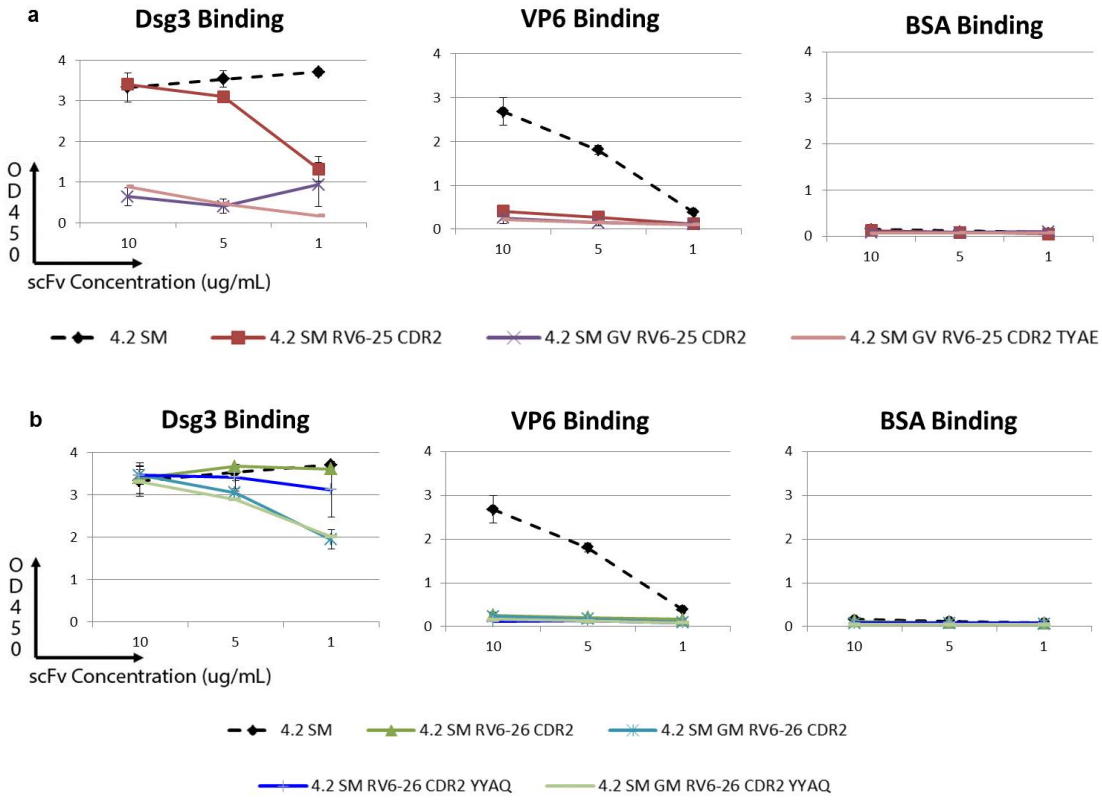
Black: 4.2 SM mutations **Blue**: RV6-25 mutations **Pink**: RV6-26 mutations

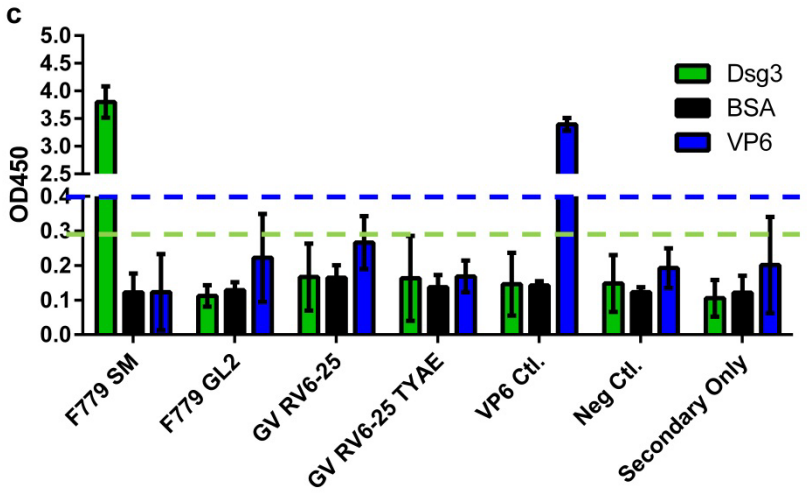
Figure 3-3. Outline of CDR2 mutagenesis experiments

(a) To test whether mutations in an around the CDR2 play a role in VH1-46 cross-reactivity, we selected two germline (GL) constructs, PVE4-8 GL and F779GL2, and one somatically mutated (SM) construct, 4.2 SM. These three VH1-46 backbones were then mutagenized in a step-wise fashion with somatic mutations from RV6-25 and RV6-26.

(b) Amino acid sequences of the CDR2 mutants. Black underlined residues indicate mutations found in 4.2 SM. Blue underlined residues indicate mutations found in RV6-25. Pink underlined residues indicate mutations found in RV6-26.

Figure 3-4. Somatic mutations in and around the VH1-46 CDR2 rarely confer cross-reactivity





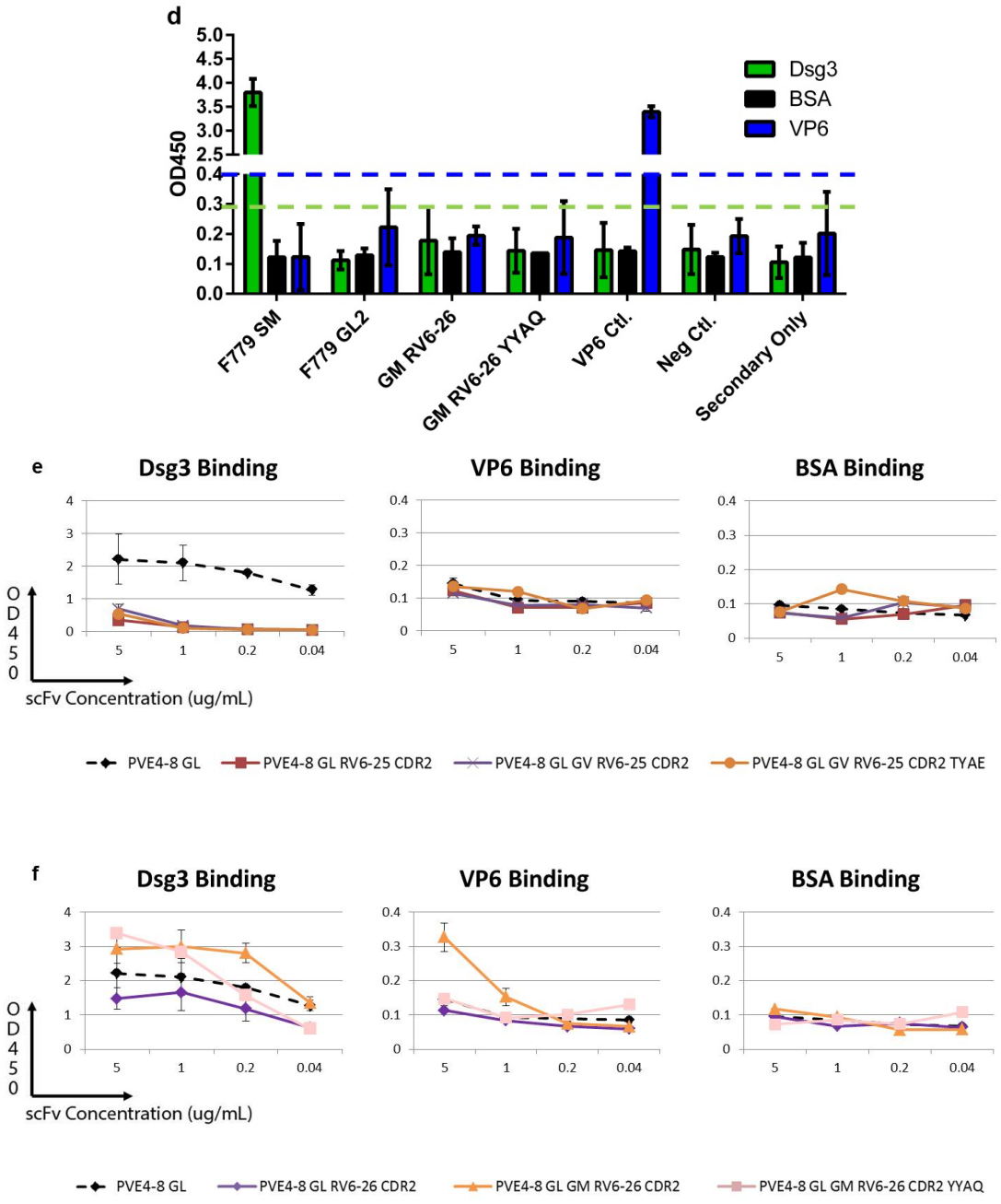


Figure 3-4. Somatic mutations in and around the VH1-46 CDR2 rarely confer cross-reactivity

(a-f) CDR2 mutants were subjected to Dsg3, VP6, and BSA ELISAs over multiple concentrations. Blue dashed line indicates VP6 cutoff value of 0.423. Green dashed line indicates Dsg3 cutoff value of 0.307. VP6 Ctl. is RV6-26. Neg Ctl. is an mAb against an irrelevant antigen. Error bars indicate SEM. Data are representative of two independent replicates.

VH1-46 Antibody	Baseline Antigen Reactivity		Mutations Added	
			RV6-25	RV6-26
4.2 SM	Dsg3	+	Reduction	Reduction
	VP6	+	Reduction	Reduction
F779 GL	Dsg3	-	No Change	No Change
	VP6	-	No Change	No Change
PVE4-8 GL	Dsg3	+	Reduction	Increase
	VP6	+/-	Reduction	Increase

Table 3-III. Summary of CDR mutagenesis experiments

Alterations in reactivity to Dsg3 and VP6 are displayed for the CDR2 mutants tested.

PV1c IgM	
VH Family	# Clones
IGHV1-18	1
IGHV1-2	1
IGHV1-69	1
IGHV2-5	1
IGHV3-21	1
IGHV3-23	5
IGHV3-30	1
IGHV3-33	2
IGHV3-48	2
IGHV4-59	1
IGHV4-61	1
IGHV5-51	5
TOTAL	22
Duplicate Clones	0
Single Clones	22

PV8 IgM	
VH Family	# Clones
IGHV1-18	1
IGHV1-2	2
IGHV1-69	1
IGHV2-5	5
IGHV3-21	1
IGHV3-33	2
IGHV4-34	8
IGHV4-59	2
IGHV4-61	4
TOTAL	26
Duplicate Clones	2
Single Clones	24

PV16 IgM	
VH Family	# Clones
IGHV1-18	2
IGHV1-2	3
IGHV1-46	1
IGHV1-69	6
IGHV1-8	1
IGHV2-26	1
IGHV3-11	4
IGHV3-13	1
IGHV3-15	1
IGHV3-21	1
IGHV3-23	3
IGHV3-30	8
IGHV3-33	1
IGHV3-48	2
IGHV3-53	1
IGHV3-7	1
IGHV3-74	1
IGHV4-31	1
IGHV4-39	1
IGHV5-51	3
TOTAL	43
Duplicate Clones	0
Single Clones	43

CH IgM	
VH Family	# Clones
IGHV1-2	2
IGHV1-46	1
IGHV1-69	2
IGHV1-8	1
IGHV2-5	1
IGHV3-23	1
IGHV3-48	1
IGHV3-66	1
IGHV4-31	1
IGHV4-34	3
IGHV4-39	4
IGHV5-51	1
TOTAL	19
Duplicate Clones	4
Single Clones	15

Table 3-IV. Diversity in VH distribution across the IgM APD libraries before selection.

A total of 40-60 clones were selected from the unpanned IgM APD libraries. Functional reads were analyzed for VH gene usage and clonality.

a

Clone	GL VH gene	GL DH gene	GL JH gene	CDR1	CDR2	CDR3	ELISA Reactivity				Dsg3 IF Reactivity
							Dsg3	VP6	BSA	Hep2	
PV1c											
PV1c IgM D3-2	IGHV1-46*03	IGHD1-1*01inv	IGHJ4*02	GYTFTSYY	INPSGGST	ARDSSLAFDY	+	+	-	ND	+
PV1c IgM D3-3	IGHV1-46*03	IGHD3-16*01	IGHJ4*02	GYTFTSYY	INPSGGST	AREERDLFLHY	+	+	-	ND	+
PV1c IgM D3-4	IGHV3-30*18	IGHD1-20*01	IGHJ4*02	GFTFSSYG	ISYDGSNK	AKGNGAPLDY	+	-	-	ND	ND
PV1c IgM D3-6	IGHV1-46*03	IGHD3-22*01	IGHJ3*02	GYTFTSYY	INPSGGST	ARDDGLSAFDI	+	-	-	ND	+
PV1c IgM D3-12	IGHV1-46*03	IGHD2-2*01	IGHJ4*02	GYTFTSYY	INPSGGST	ARDMLPAVGDY	+	-	-	ND	+
PV1c IgM D3-14	IGHV1-18*01	IGHD3-10*01	IGHJ6*02	GYTFTSYG	ISAYNGNT	ATHTGRYGMDV	+	-	-	ND	ND
PV1c IgM D3-22	IGHV3-7*01	IGHD2-2*01inv	IGHJ6*02	GFTFSSYW	IKQDGSEK	ARVGRGTGTIDYYYGMDV	+	-	-	ND	ND
PV1c IgM D3-24	IGHV1-18*01	IGHD5-12*01	IGHJ6*02	GYTFTSYG	ISAYNGNT	ARGGDIVATIGYYYGMDV	+	-	-	ND	ND
PV1c IgM DVD-1	IGHV3-64*01	IGHD3-10*01	IGHJ6*02	GFTFSSYA	ISSNGGST	ARGPVRGVIDQADYYYYYGTDV	+	+	-	ND	-
PV1c IgM DVD-5	IGHV1-46*03	IGHD2-2*02	IGHJ4*02	GYTFTSYY	INPSGGST	ARESCSSTSCYFDY	+	+	-	ND	+
PV1c IgM DVDV-1	IGHV1-46*03	IGHD1-26*01	IGHJ4*02	GYTFTSYY	INPSGGST	ARDSGSLSFDY	+	+	-	ND	+
PV1c IgM DVDV-3	IGHV4-28*03	IGHD4-17*01	IGHJ4*02	GYTFTSYY	INPSGGST	ARIDYGIDY	+	-	-	ND	ND
PV1c IgM DVDV-6	IGHV1-46*03	IGHD5-5*01	IGHJ4*02	GYTFTSYY	INPSGGST	ARDFGRGYSYGYPGHFDY	-	+	-	ND	-
PV1c IgM DVDV-7	IGHV1-46*03	IGHD1-26*01	IGHJ4*02	GYTFTSYY	INPSGGST	ARDRELALDY	+	+	-	ND	+
PV1c IgM DVDV-8	IGHV1-46*03	IGHD6-13*01	IGHJ4*02	GYTFTSYY	INPSGGST	ARDDSSSLDY	+	+	-	ND	+

b

Clone	GL VL gene	GL JL gene	CDR1	CDR2	CDR3	ELISA Reactivity				Dsg3 IF Reactivity
						Dsg3	VP6	BSA	Hep2	
PV1c										
PV1c IgM D3-2	IGLV2-8*01	IGLJ1*01	SSDVGGYNY	EVS	SSYAGSNKHV	+	+	-	ND	+
PV1c IgM D3-3	IGLV2-8*01	IGLJ7*01	SSDVGSYNL	EVT	FSYAGDNNFV	+	+	-	ND	+
PV1c IgM D3-4	IGLV6-57*01	IGLJ3*02	SGSIASNY	EDN	QSYDSSTWV	+	-	-	ND	ND
PV1c IgM D3-6	IGLV2-23*01	IGLJ3*01	SGDVGRYNL	EGS	FSYSGHNTGI	+	-	-	ND	+
PV1c IgM D3-12	IGLV2-8*01	IGLJ3*02	SSDIGGYNY	EVT	LSYAGNNNFI	+	-	-	ND	+
PV1c IgM D3-14	IGLV7-43*01	IGLJ3*02	TGAVTSGYY	STS	LLYYGGAQLV	+	-	-	ND	ND
PV1c IgM D3-22	IGLV3-1*01	IGLJ3*02	KLADKY	QDD	QAWDSTTVMV	+	-	-	ND	ND
PV1c IgM D3-24	IGLV2-14*04	IGLJ3*01	SSDVGGYNY	DVS	SSYTSSSTVV	+	-	-	ND	ND
PV1c IgM DVD-1	IGLV1-44*01	IGLJ3*02	SSNIGSNT	SNN	AAWDDSLNGRV	+	+	-	ND	-
PV1c IgM DVD-5	IGLV3-21*02	IGLJ3*01	NIGSKS	DDS	QVWDSSSDVV	+	+	-	ND	+
PV1c IgM DVDV-1	IGLV2-8*01	IGLJ3*01	SSDVGGYNY	EVS	SSYAGSNNFV	+	+	-	ND	+
PV1c IgM DVDV-3	IGLV1-40*01	IGLJ3*02	SSNIGAGYD	GNS	QSYDSSLSARV	+	-	-	ND	ND
PV1c IgM DVDV-6	IGKV3D-11*01	IGKJ4*01	QSVSSY	DAS	QQRSNWPLT	-	+	-	ND	-
PV1c IgM DVDV-7	IGLV2-8*01	IGLJ7*01	SSDVGAYNY	EVT	SSYAGSNTVV	+	+	-	ND	+
PV1c IgM DVDV-8	IGLV2-23*01	IGLJ3*02	SSDIGSYDL	EDT	FSYAGKSVFV	+	+	-	ND	+

Table 3-V. PV1c IgM APD clones

APD libraries were subjected to four rounds of either single- or double-antigen selection. Germline (GL) assignments are displayed, along with CDR sequences. D3/4 indicates isolation from rounds 3 or 4 Dsg3-based selection, respectively. DVD/DVDV indicates isolation from rounds 3 or 4 Dsg3/VP6-based selection, respectively. Reactivity to various antigens was also tested by ELISA and immunofluorescence (IF). ND indicates not determined. Clones highlighted in gray also were isolated from double-antigen selection.

(a) Heavy chains
(b) Light chains

a

Clone	GL VH gene	GL DH gene	GL JH gene	CDR1	CDR2	CDR3	ELISA Reactivity				Dsg3 IF Reactivity
							Dsg3	VP6	BSA	Hep2	
PV8											
PV8 IgM D3-2	IGHV4-34*01	IGHD4-4*01	IGHJ3*02	GGSFSGYY	INHSGST	ARAGNYGNFDGFDI	+	-	-	ND	ND
PV8 IgM D3-4	IGHV2-5*02	IGHD6-19*01	IGHJ4*02	GFSLTTTGVG	IFWDDDK	AHTGWLGFFDY	+	-	-	ND	ND
PV8 IgM D3-6	IGHV3-33*01	IGHD4-17*01	IGHJ4*02	GFTFSSYG	IRYDGSNK	ANWRPTGPTYFDS	+	-	-	ND	ND
PV8 IgM D3-13	IGHV1-46*03	IGHD1-26*01	IGHJ5*02	GYTFTSYY	INPSGGST	ARGGIVGATTGGNNWFDP	+	-	-	ND	ND
PV8 IgM D3-19	IGHV5-51*01	IGHD4-17*01	IGHJ4*02	GYSFTSYW	IYPGDSDT	ARLRYGDYGGAFDY	+	-	-	ND	ND
PV8 IgM D3-27	IGHV4-31*03	IGHD4-17*01	IGHJ3*02	GDSISSGGYS	IYHSGNT	AGYGIDDAFDI	+	-	-	ND	ND
PV8 IgM D3-36	IGHV7-4-1*02	IGHD6-25*01	IGHJ3*02	GYTFTSYG	INTNTGNP	ARGRDAFDI	+	-	-	ND	ND
PV8 IgM D4-11	IGHV5-51*01	IGHD5-5*01	IGHJ4*02	GYSFTSYW	IYPGDSDI	ARGPDTASFDY	+	-	-	ND	ND
PV8 IgM DVD-7	IGHV1-69*06	IGHD1-1*01	IGHJ5*02	GYTFTSYG	INPNSGGT	ARNPPATGSFDP	+	+	+	-	-
PV8 IgM DVDV-5	IGHV1-8*01	IGHD3-16*01	IGHJ5*02	GYTFTSYD	MNPNSGNT	ARGGWFDP	ND	ND	ND	ND	ND

b

Clone	GL VL gene	GL JL gene	CDR1	CDR2	CDR3	ELISA Reactivity				Dsg3 IF Reactivity
						Dsg3	VP6	BSA	Hep2	
PV8										
PV8 IgM D3-2	IGLV1-51*01	IGLJ3*01	SSNIGSSY	DDS	GTWDSSLSSVV	+	-	-	ND	ND
PV8 IgM D3-4	IGLV3-21*02	IGLJ3*01	NIGSKS	DDD	QVWDTTSDRVL	+	-	-	ND	ND
PV8 IgM D3-6	IGKV3-20*01	IGLJ3*01	QSVNSNS	SAS	QQYGGSPFT	+	-	-	ND	ND
PV8 IgM D3-13	IGLV1-36*01	IGLJ3*01	SSNIGSNS	YDD	AAWDDSLSGVV	+	-	-	ND	ND
PV8 IgM D3-19	IGLV2-14*02	IGLJ3*02	SSDVGTYNL	EVS	SSYAGNNNLL	+	-	-	ND	ND
PV8 IgM D3-27	IGKV1-39*01	IGKJ4*01	QSISSY	TAS	QQSYSTPLT	+	-	-	ND	ND
PV8 IgM D3-36	IGKV1-33*01	IGKJ5*01	QDIRNY	DAS	QQYDNLPIT	+	-	-	ND	ND
PV8 IgM D4-11	IGLV7-43*01	IGLJ3*01	TGAVTSGYY	STS	LLYYGGHVV	+	-	-	ND	ND
PV8 IgM DVD-7	IGKV1-39*01	IGKJ5*01	QSISSY	AAS	QQAYSFPIT	+	+	+	-	-
PV8 IgM DVDV-5	IGLV7-46*01	IGLJ7*01	TGAVTSGHY	DTT	LLSYGAVEV	ND	ND	ND	ND	ND

Table 3-VI. PV8 IgM APD clones

APD libraries were subjected to four rounds of either single- or double-antigen selection. Germline (GL) assignments are displayed, along with CDR sequences. D3/4 indicates isolation from rounds 3 or 4 Dsg3-based selection, respectively. DVD/DVDV indicates isolation from rounds 3 or 4 Dsg3/VP6-based selection, respectively. Reactivity to various antigens was also tested by ELISA and immunofluorescence (IF). ND indicates not determined. One clone was unable to be produced as recombinant protein, and is listed as ND for all antigens.

(a) Heavy chains
(b) Light chains

a

							ELISA Reactivity				Dsg3 IF
Clone	GL VH gene	GL DH gene	GL JH gene	CDR1	CDR2	CDR3	Dsg3	VP6	BSA	Hep2	Reactivity
PV16											
PV16 IgM D3-1	IGHV4-39*01	IGHD1-26*01	IGHJ5*02	GGSISSSSYY	IYYSGST	ARQGRGYSGSYYEILWFDP	+	-	-	ND	ND
PV16 IgM D3-5	IGHV3-7*01	IGHD3-3*02inv	IGHJ4*02	GFTFSSYW	IKQDGSEK	ARSKRGFGY	+	+	-	+	-
PV16 IgM D3-6	IGHV2-5*01	IGHD2-15*01	IGHJ1*01	GFSLSTSGVG	IYWNDDK	AHSSGYCSGGSCYPSFAEYFQH	+	-	-	ND	ND
PV16 IgM D3-7	IGHV4-34*01	IGHD3-10*01	IGHJ5*02	GGSFSDYY	INHRGST	ARTRGTGSGSKKGFDP	+	-	-	ND	ND
PV16 IgM D3-8	IGHV5-51*01	IGHD5-24*01inv	IGHJ2*01	GYSFTSYW	IYPGDSDT	ARWAPSLGGRNWYFDL	+	-	-	ND	ND
PV16 IgM D3-9	IGHV1-69*01	IGHD3-22*01	IGHJ5*02	GGTFSSYA	IIPIFGTA	AMDYYDSSGYREFVYWFDP	ND	ND	ND	ND	ND
PV16 IgM D3-11	IGHV5-51*01	IGHD3-16*01	IGHJ3*02	GYSFTSYW	IYPGDSDT	ARVGHGGHFDI	+	-	-	ND	ND
PV16 IgM D3-12	IGHV3-7*01	IGHD2-8*02	IGHJ5*02	GFTFSSYW	IKQDGSEK	AREPRYFDWFSGWFDP	+	-	-	ND	ND
PV16 IgM D3-14	IGHV3-64*02	IGHD3-16*01	IGHJ4*03	FTFSNAW	ISSNGGTT	VKDFMFGVLTRGSHPPGI	+	-	-	ND	ND
PV16 IgM D3-16	IGHV3-7*01	IGHD4-23*01	IGHJ4*02	GFTFSSYW	IKQDGSEK	AREGGFYYGDNFDY	+	-	-	ND	ND
PV16 IgM D3-23	IGHV3-48*03	IGHD5-12*01	IGHJ6*02	GFTFSSYE	ISSSGSTI	AREATLEDYYYYGMDV	+	-	-	ND	ND
PV16 IgM D4-1	IGHV3-66*01	IGHD3-10*01	IGHJ4*02	GFTVSSNY	IYSGGST	WARRLWNGIDY	+	-	-	ND	ND
PV16 IgM D4-3	IGHV1-46*03	IGHD3-9*01	IGHJ4*02	GYTFTSYY	INPSGGST	ARDLGIVPGDY	+	-	-	ND	+
PV16 IgM D4-19	IGHV3-23*01	IGHD3-9*01	IGHJ4*02	GFTFSSYA	ISGSGGST	AKPNIYYFDY	+	-	-	ND	ND
PV16 IgM D4-24	IGHV1-46*03	IGHD6-19*01inv	IGHJ4*02	GYTFTSYY	INPSGGST	ARDAIPATFDY	+	-	-	ND	+
PV16 IgM DVD-4	IGHV3-30*18	IGHD3-22*01inv	IGHJ3*02	GFTFSSYG	ISYDGSNK	ARDQGI	ND	ND	ND	ND	ND
PV16 IgM DVD-8	IGHV3-11*01	IGHD1-26*01	IGHJ5*02	GFTFSDYY	ISSSGSTI	ASDSPGDYWGPGNPGHRLLR	+	+	+	-	-
PV16 IgM DVD-16	IGHV5-51*01	IGHD2-2*01	IGHJ5*02	GYSFTSYW	IYPGDSDT	ARRRYCSSTSCYDWFDP	-	+	-	ND	ND
PV16 IgM DVD-18	IGHV3-13*01	IGHD3-22*01	IGHJ5*02	GFTFSSYD	IGTAGDT	ARDAYDSSGYYYSDY	ND	ND	ND	ND	ND
PV16 IgM DVDV-15	IGHV3-15*01	IGHD1-20*01	IGHJ3*01	GFTFSNAW	IKCKTDGGTT	TTADRRTGTTKALDV	+	+	+	+	ND
PV16 IgM DVDV-16	IGHV3-23*01	IGHD1-26*01	IGHJ4*02	GFTFNSYG	ISGSGGST	ARSYSGSYAWGPGNPGHRLLR	ND	ND	ND	ND	ND
PV16 IgM DVDV-23	IGHV3-30*18	IGHD3-10*02	IGHJ6*02	GFTFSSYG	ISYDGSNK	AKDRRRKGMDV	+	+	+	+	-

b

						ELISA Reactivity				Dsg3 IF
Clone	GL VL gene	GL JL gene	CDR1	CDR2	CDR3	Dsg3	VP6	BSA	Hep2	Reactivity
PV16										
PV16 IgM D3-1	IGLV3-1*01	IGLJ3*01	KLGDKY	QDS	QAWDNSTVV	+	-	-	ND	ND
PV16 IgM D3-5	IGLV6-57*01	IGLJ3*01	SGSIASNY	EDN	QSYDSSNPHVV	+	+	-	+	-
PV16 IgM D3-6	IGLV1-40*01	IGLJ3*01	RSNIGASYD	SNT	QSYDNTLSGSSVV	+	-	-	ND	ND
PV16 IgM D3-7	IGKV3-20*01	IGKJ3*01	QSFSRY	GAS	QQYGSVPFT	+	-	-	ND	ND
PV16 IgM D3-8	IGLV3-1*01	IGLJ3*01	KLGDKY	QDN	QAWDSSTVV	+	-	-	ND	ND
PV16 IgM D3-9	IGLV8-61*01	IGLJ3*02	SGSVSSQYY	NTN	VLYLGRSTWV	ND	ND	ND	ND	ND
PV16 IgM D3-11	IGLV1-47*01	IGLJ7*01	SSNIGSNY	RNN	SAWDHSLNALL	+	-	-	ND	ND
PV16 IgM D3-12	IGLV6-57*01	IGLJ3*01	SGDIASDY	EDN	QSYDDNTVV	+	-	-	ND	ND
PV16 IgM D3-14	IGLV1-44*01	IGLJ3*02	SSNIGRNT	SNN	AAWDDSLNGWL	+	-	-	ND	ND
PV16 IgM D3-16	IGLV3-21*03	IGLJ3*01	NIGSKS	DDS	QAWDSSTAV	+	-	-	ND	ND
PV16 IgM D3-23	IGLV3-21*03	IGLJ3*01	NIGSKS	DDS	QVWDSSSAGVV	+	-	-	ND	ND
PV16 IgM D4-1	IGLV1-51*01	IGLJ3*02	SSNIGNNY	DNN	GTWDSSLSAWV	+	-	-	ND	ND
PV16 IgM D4-3	IGLV2-8*01	IGLJ3*01	SSDVGGYNY	EVS	SSYAGSNNLL	+	-	-	ND	+
PV16 IgM D4-19	IGLV3-1*01	IGLJ1*01	KLGDKY	QDS	QAWDSSTYV	+	-	-	ND	ND
PV16 IgM D4-24	IGLV2-8*01	IGLJ1*01	SSDVGGYNY	EVS	SSYAGSNNYV	+	-	-	ND	+
PV16 IgM DVD-4	IGLV1-51*01	IGLJ3*01	SSNIGNNY	DNN	GTWDSSLSAVV	ND	ND	ND	ND	ND
PV16 IgM DVD-8	IGKV2-28*01	IGKJ5*01	QSLLHSNGNNY	LGS	MQALQTPPT	+	+	+	-	-
PV16 IgM DVD-16	IGLV2-14*01	IGLJ3*01	SSDVGGYNY	EVS	SSYTSSSTVV	-	+	-	ND	ND
PV16 IgM DVD-18	IGKV1-39*01	IGKJ3*01	QDVNYY	DAS	QQFDNVPYT	ND	ND	ND	ND	ND
PV16 IgM DVDV-15	IGLV1-44*01	IGLJ7*01	NSNIGSNT	SNN	AAWDDSLNGRV	+	+	+	+	ND
PV16 IgM DVDV-16	IGLV2-14*04	IGLJ3*01	SSDVGSYSH	DVS	SSYTSSSTLVV	ND	ND	ND	ND	ND
PV16 IgM DVDV-23	IGLV3-21*01	IGLJ3*02	NIGTKS	QDD	QAWDSGGV	+	+	+	+	-

Table 3-VII. PV16 IgM APD clones

APD libraries were subjected to four rounds of either single- or double-antigen selection. Germline (GL) assignments are displayed, along with CDR sequences. D3/4 indicates isolation from rounds 3 or 4 Dsg3-based selection, respectively. DVD/DVDV indicates isolation from rounds 3 or 4 Dsg3/VP6-based selection, respectively. Reactivity to various antigens was also tested by ELISA and immunofluorescence (IF). Clones highlighted in gray also were isolated from double-antigen selection. ND indicates not determined. Some clones were unable to be produced as recombinant protein, and are listed as ND for all antigens.
(a) Heavy chains **(b)** Light chains

a

Clone	GL VH gene	GL DH gene	GL JH gene	CDR1	CDR2	CDR3	ELISA Reactivity				Dsg3 IF Reactivity
							Dsg3	VP6	BSA	Hep2	
CH											
CH IgM D2-4	IGHV4-34*01	IGHD2-2*01	IGHJ4*02	GGSFSGYY	INHSGST	ARACLGRLGYCSSTGPPATGHFDY	ND	ND	ND	ND	ND
CH IgM D3-1	IGHV3-23*01	IGHD4-23*01	IGHJ6*02	GFTFSSYG	ISGSGGST	ARDQNGGDYYGMDV	+	-	-	ND	+
CH IgM D3-4	IGHV3-21*01	IGHD3-22*01	IGHJ3*02	GFTFSSYS	ISSSSSYI	ARAKNYYDSSGYYYPDAFDI	+	-	-	ND	+
CH IgM D4-3	IGHV3-66*01	IGHD4-17*01	IGHJ4*02	GFTFSSYA	IYSGGST	ARAQYYGDYFDY	+	-	-	ND	+
CH IgM DVDV-3	IGHV3-23*01	IGHD4-17*01	IGHJ3*02	GFTFSNAW	ISGSGGST	ARADGAAFDI	+	+	+	+	-

b

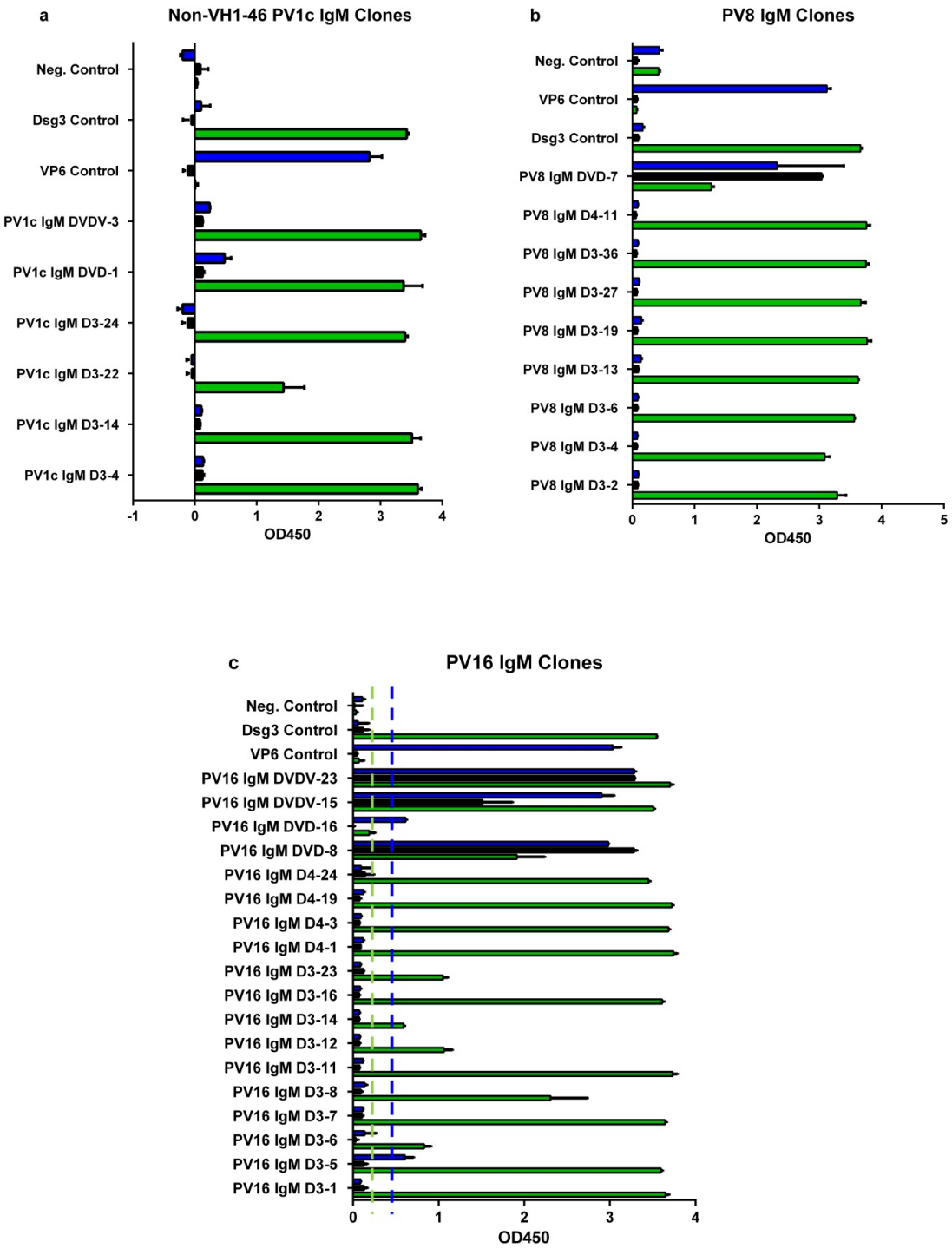
Clone	GL VL gene	GL JL gene	CDR1	CDR2	CDR3	ELISA Reactivity				Dsg3 IF Reactivity
						Dsg3	VP6	BSA	Hep2	
CH										
CH IgM D2-4	IGKV5-2*01	IGKJ1*01	QDIDDD	EAT	LQHDNFPLT	ND	ND	ND	ND	ND
CH IgM D3-1	IGKV1-39*01	IGKJ4*01	QSISSY	AAS	QQSYSTPT	+	-	-	ND	+
CH IgM D3-4	IGKV4-1*01	IGKJ1*01	QSVLYSSNNKNY	WAS	QQYGSSPWT	+	-	-	ND	+
CH IgM D4-3	IGLV1-47*01	IGLJ3*01	SSNIGSNY	RNN	AAWDDSLSGVV	+	-	-	ND	+
CH IgM DVDV-3	IGKV1-39*01	IGKJ5*01	QGISSY	AAS	QQSYSTPIA	+	+	+	+	-

Table 3-VIII. CH IgM APD clones

APD libraries were subjected to four rounds of either single- or double-antigen selection. Germline (GL) assignments are displayed, along with CDR sequences. D3/4 indicates isolation from rounds 3 or 4 Dsg3-based selection, respectively. DVD/DVDV indicates isolation from rounds 3 or 4 Dsg3/VP6-based selection, respectively. Reactivity to various antigens was also tested by ELISA and immunofluorescence (IF). ND indicates not determined. One clone was unable to be produced as recombinant protein, and is listed as ND for all antigens.

(a) Heavy chains
(b) Light chains

Figure 3-5. A majority of non-VH1-46 IgM heavy chains do not demonstrate cross-reactivity



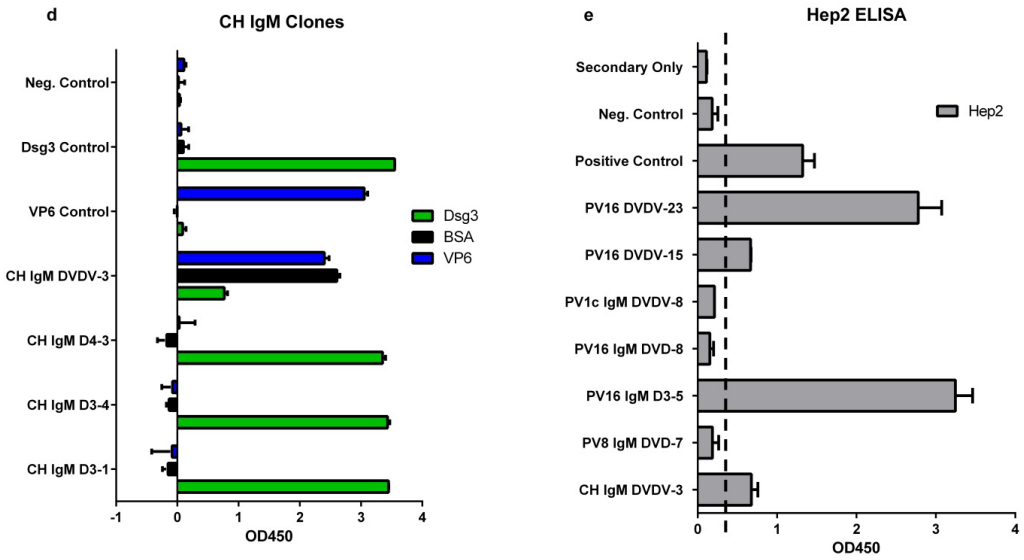


Figure 3-5. A majority of non-VH1-46 IgM heavy chains do not demonstrate cross-reactivity

(a-e) IgM APD libraries were single- and double-antigen screened. Clones were randomly isolated and validated for Dsg3 reactivity. Those that were positive were purified as scFv and subjected to ELISA against various antigens. In some cases, clones were unable to purified as soluble scFv, and are reported as not determined (ND). Green dashed line indicates Dsg3 cutoff value of 0.209. Blue dashed line indicates VP6 cutoff value of 0.409. Gray dashed line indicates Hep2 cutoff value of 0.362. Dsg3 control is clone (D31)2/29. VP6 control is RV6-26. Neg. Control is a mAb against an irrelevant antigen. Commercial positive control is displayed in the HEp2 ELISA. Error bars indicate SEM. Data represent two independent experiments.

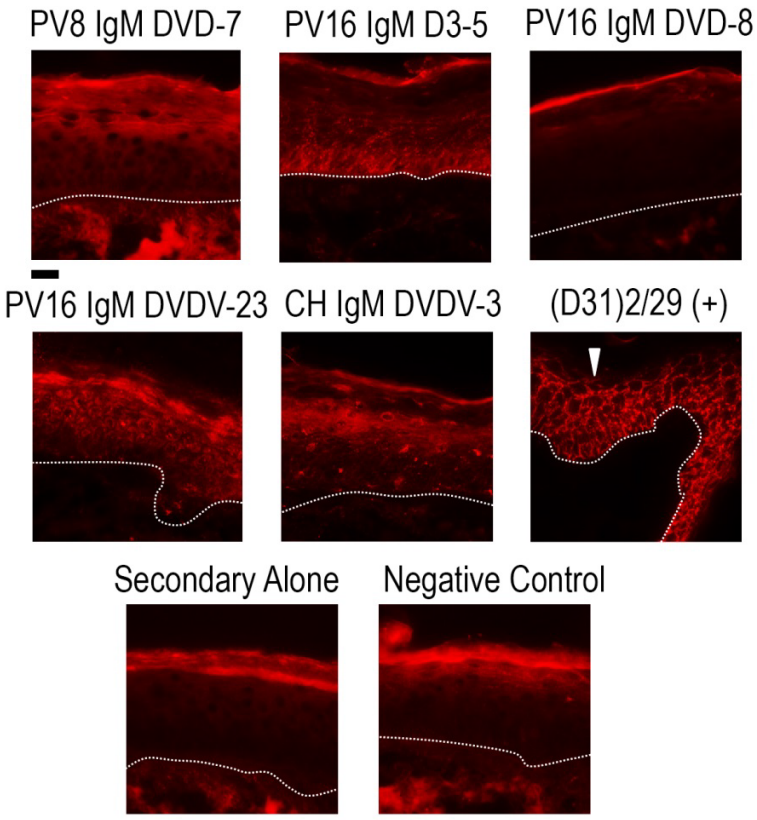
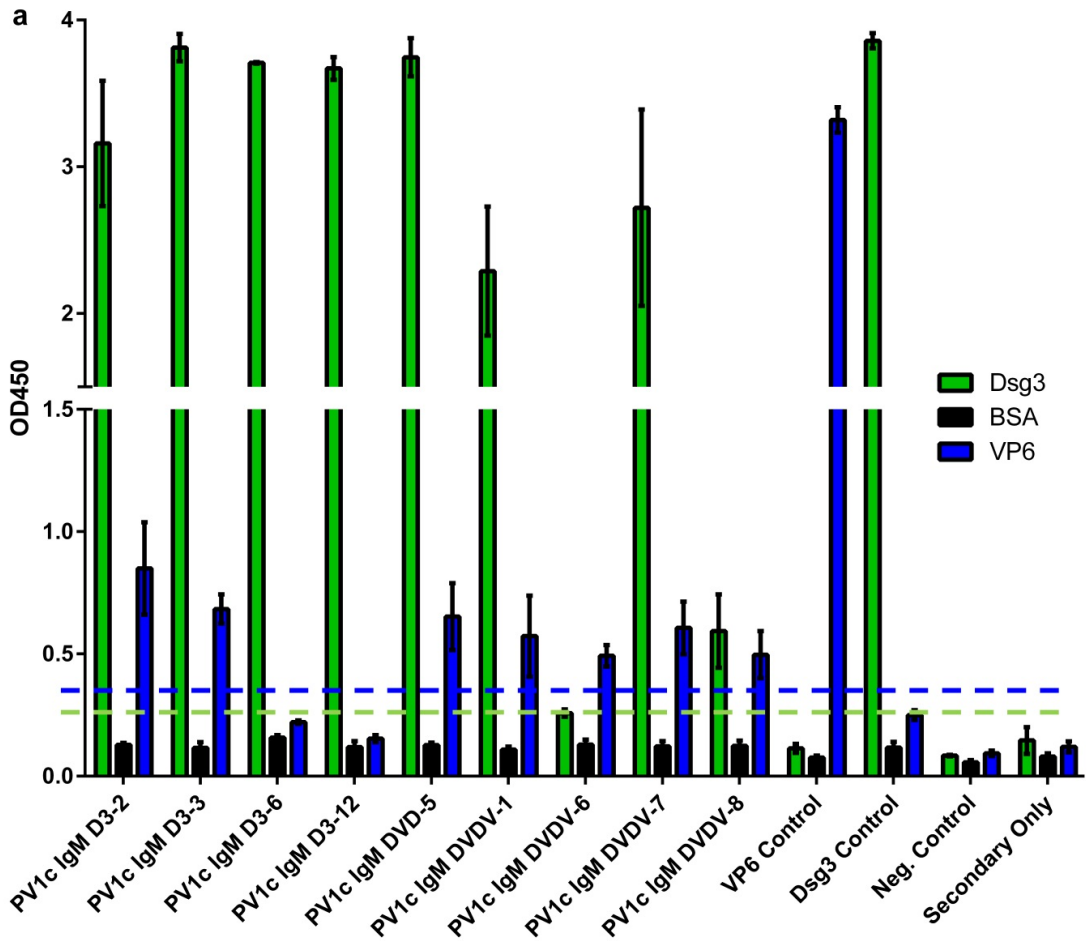


Figure 3-6. Polyreactive clones do not bind Dsg3 in human skin

All polyreactive clones based on ELISA were subjected to immunofluorescence on human skin. Negative Control is a mAb against an irrelevant antigen. White dashed line indicates dermal-epidermal junction. White arrowhead indicates surface staining of human skin. Scale bar, 20 μM. Data are representative of 1-2 experiments tested at multiple concentrations.

Figure 3-7. Validation of PV1c VH1-46 clones



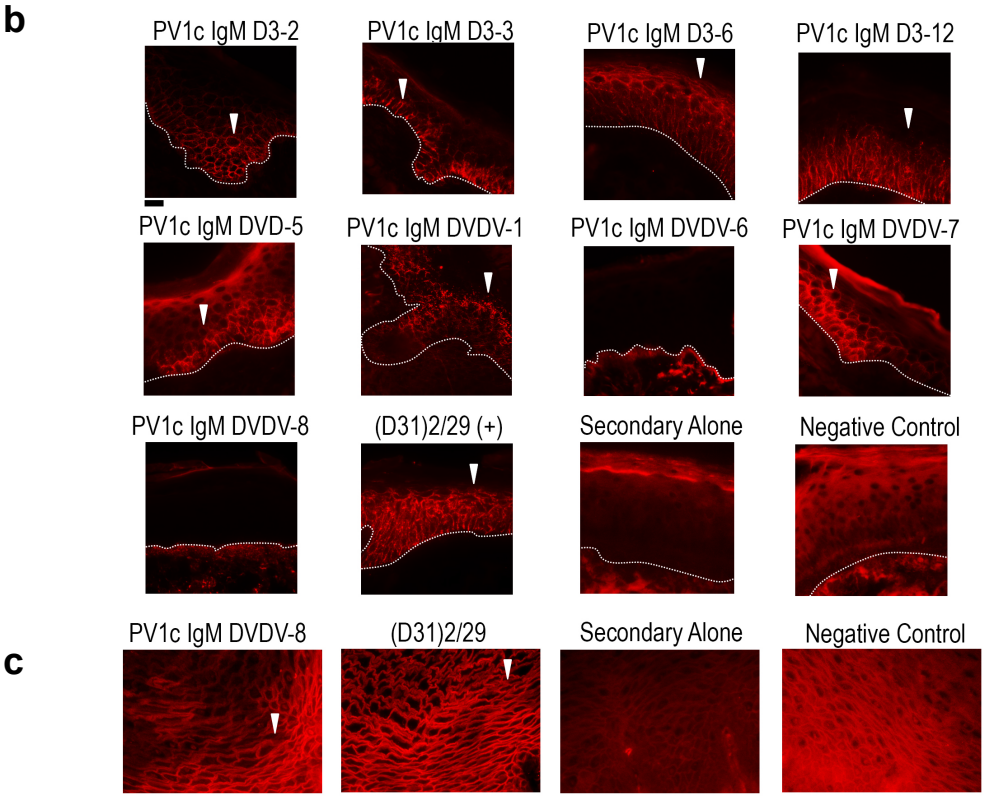


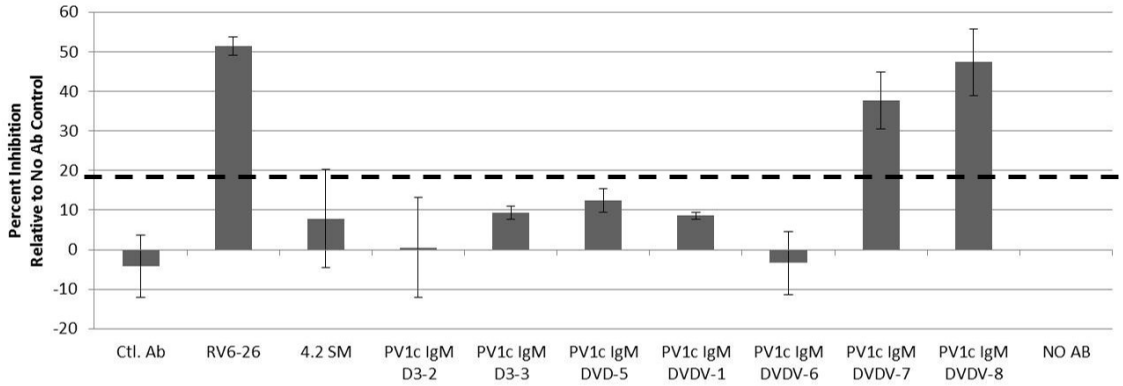
Figure 3-7. Validation of PV1c VH1-46 clones

(a) VH1-46 PV1c IgM clones were tested by ELISA. Dsg3 control is clone (D31)2/29. VP6 control is RV6-26. Neg. Control is a mAb against an irrelevant antigen. Green dashed line indicates Dsg3 cutoff value of 0.268. Blue dashed line indicates VP6 cutoff value of 0.358. Error bars indicate SEM. Data are representative of two or three independent experiments.

(b) VH1-46 PV1c IgM clones were tested by immunofluorescence against human skin. White dashed line indicates dermal-epidermal junction. White arrowhead indicates surface staining of human skin.

(c) PV1c IgM DVDV-8 subjected to immunofluorescence on monkey esophagus. Negative Control is a mAb against an irrelevant antigen. Scale bar, 20 μM. Data are representative of 1-2 experiments tested at multiple concentrations.

a



b

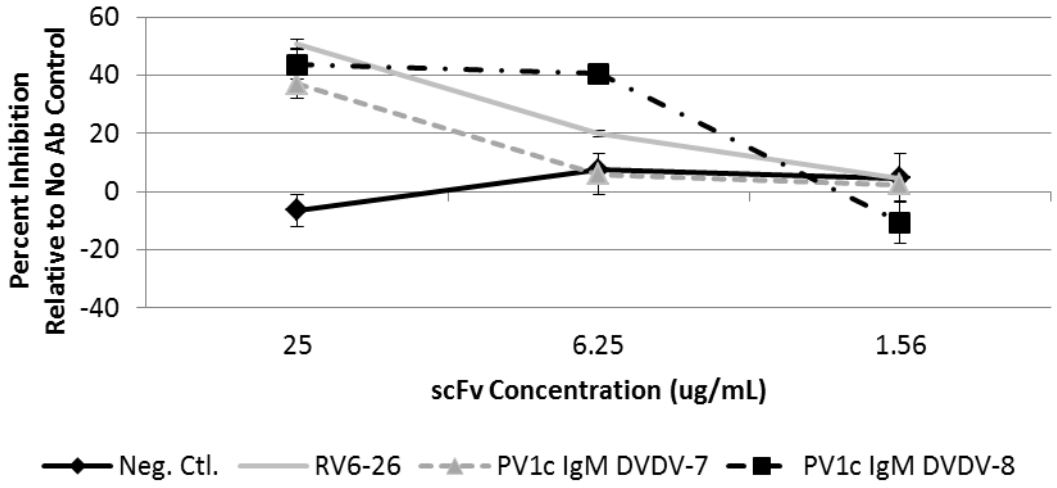


Figure 3.8. A subset of cross-reactive VH1-46 heavy chains inhibit rotavirus replication *in vitro*

(a-b) scFv were incubated with rotavirus double-layered particles (DLPs). MA104 cells were then transfected with the mixture, incubated overnight, and subsequently stained for rotavirus foci after fixation. Foci were quantitated using ImageJ. Black dashed line indicates cutoff value of 19.88 for positive inhibition.

(a) Each scFv was tested at a concentration of 25 ug/mL. Data are representative of three or four replicates.

Neg. Ctl. is a mAb against an irrelevant antigen. Y axis indicates level of inhibition as a percent of the no antibody control. Error bars indicate SEM. Data are representative of two independent experiments.

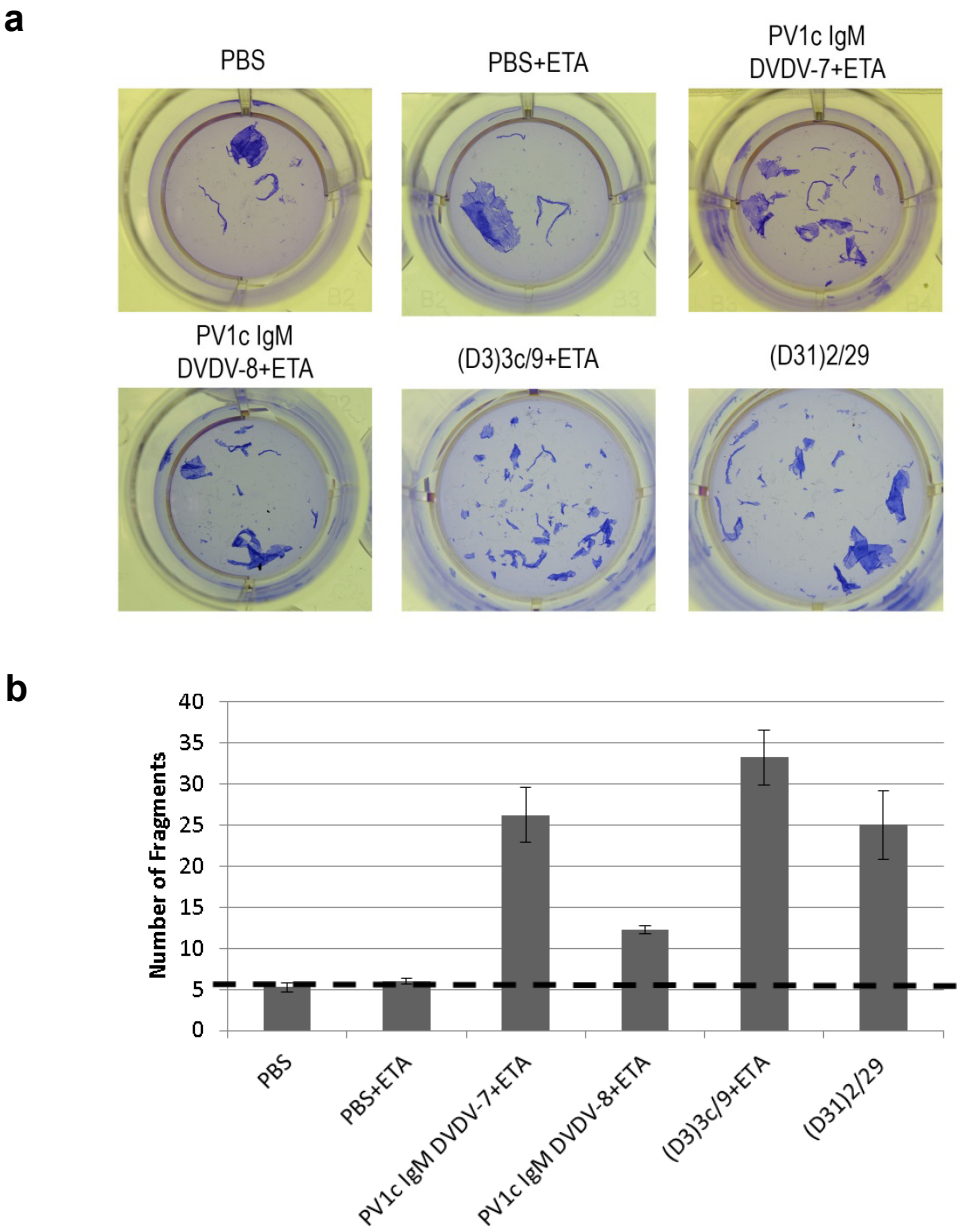


Figure 3-9. PV1c IgM VH1-46 DVDV-7 and DVDV-8 induce keratinocyte dissociation

(a-b) Confluent primary human keratinocytes were incubated with scFv overnight and subjected to manual dissociation. Fragments were quantitated using ImageJ. Black dashed line indicates cutoff value of 6.75. Error bars indicate SEM. Data are representative of two independent experiments.

CHAPTER 4: DISCUSSION AND FUTURE DIRECTIONS

4.1 Summary of findings

We have established that in the four patient cohort we studied, the single shared characteristic in their anti-Dsg3 mAb repertoire is the utilization of VH1-46 in the anti-Dsg3 B cell repertoire. This is likely due to the ability of these VH1-46 mAbs to bind Dsg3 in their unmutated state, or with a very low number of somatic mutations leading to acidic amino acid residues (Chapter 2). Based on these findings, we hypothesized that due to shared VH1-46 gene usage in the autoimmune response to Dsg3 and the immune response to the rotavirus protein VP6, that these Ab responses may be connected to one another by a cross-reactive VH1-46 common ancestor. We show in Chapter 3 that cross-reactivity in the IgG compartment is rare due to a divergence in amino acid characteristics between the anti-Dsg3 and anti-VP6 VH1-46 responses. Evidence of cross-reactive VH1-46 IgM heavy chains was described in one PV patient in remission after rituximab, and this was not observed in three additional libraries; two from patients with active disease, and one healthy individual. Interestingly, two of these cross-reactive VH1-46 heavy chains are able to both inhibit the replication of rotavirus and keratinocyte adhesion *in vitro*, and may point to why these Dsg3-reactive heavy chains are able to contribute to the B cell repertoire despite being reactive to self in certain individuals. Thus, our work has established a potential reason why VH1-46 may be enriched in certain PV patients.

4.2 Key implications of this work

4.2.1 VH1-46 utilization is observed in the Dsg3-reactive Ab repertoires across all patients analyzed.

The study described in Chapter 2, while not the first to describe the autoAb repertoire in PV patients[281, 283], is the most comprehensive in terms of encompassing all B cell subsets in the peripheral blood. In addition, the utilization of multiple methods of Ab repertoire cloning underscores the validity of the VH1-46 phenotype we observe in the anti-Dsg3 Ab repertoires of our four patient cohort. Lastly, we provide strong evidence of these VH1-46 Abs in contributing to the pathogenesis of PV via *ex vivo* human skin injection.

4.2.2 A subset of VH1-46 mAbs are able to bind Dsg3 upon reversion of somatic mutations.

Somatic mutation analyses revealed a relative dearth of somatic mutations in the VH1-46 Abs compared to non-VH1-46 Abs, leading us to the hypothesis that perhaps these VH1-46 Abs do not largely depend on somatic mutation for Dsg3 reactivity. Germline (GL) reversion experiments confirmed that three of the five VH1-46 Abs maintained the ability to bind Dsg3 upon reversion, compared to zero of five non-VH1-46 Abs. This GL reactive phenotype is also in direct contrast to four non-VH1-46 Abs isolated from two

other PV patients[281], wherein all four also required somatic mutations in the heavy chain for Dsg3 reactivity.

4.2.3 Those VH1-46 mAbs that did require somatic mutations to bind depend on one or two somatic mutations that led to acidic amino residues.

Given that two VH1-46 Abs lost the ability to bind Dsg3 upon reversion, we wanted to better understand why this was the case. We observed an enrichment of replacement somatic mutations leading to acidic amino acid residues, and upon reintroduction of these residues into the GL Ab, we observed a partial rescue of these two non-GL-reactive Abs in terms of Dsg3 binding. Furthermore, removal of acidic residues in two GL-reactive Abs led to a complete loss of binding, suggesting that acidic residues are both necessary and partially sufficient to confer Dsg3 reactivity in the VH1-46 Abs we have tested, and can be generate by either V(D)J recombination, or somatic hypermutation.

4.2.4 Despite shared VH1-46 gene usage in the antibody responses to both Dsg3 and VP6, cross-reactivity in the IgG compartment is rare due to disparate amino acid characteristics.

The striking VH1-46 phenotype we observed in the autoAb response to Dsg3 led us to determine whether other VH1-46-biased Ab responses may play a role in the etiology of PV. The only other reported VH1-46-biased Ab response to an antigen is towards the rotavirus protein VP6. Both of the VH1-46 responses demonstrated lower levels of somatic mutations, as well as reactivity towards Dsg3 and VP6 in the absence of these somatic mutations, respectively. We hypothesized that PV could be initiated by a cross-reactive VH1-46 B cell clone. Focused testing of our Dsg3-reactive VH1-46 Abs largely did not reveal strong levels of cross-reactivity, with one of seven IgG clones being cross-reactive. In addition, screening four IgG repertoires by antibody phage display did not identify cross-reactive clones.

Further analyses of the amino acid characteristics in the Dsg3 and VP6 VH1-46 responses pointed to distinct patterns of acidic and basic residues[245], respectively. Indeed, a majority of the mutagenesis experiments conducted on the heavy chain CDR2 to introduce rotavirus-specific somatic mutations did not confer cross-reactivity to three VH1-46 clones isolated from PV patients. However, in one case, there was a moderate increase in cross-reactivity to both Dsg3 and VP6, suggesting that while rare, it is possible to heighten cross-reactivity of VH1-46 clones via somatic hypermutation.

4.2.5 A majority of VH1-46 heavy chains in the IgM compartment are cross-reactive in a PV patient in remission, but not in two patients with active disease or a healthy individual.

In order to maximize the probability of isolating a cross-reactive clone, we screened the IgM repertoires of four individuals; three PV patients, and one healthy individual. We chose a diverse cohort of individuals because the hypothesis of a cross-reactive VH1-46 clone alludes to a secondary assumption that perhaps everyone exhibits cross-reactive VH1-46 heavy chains, especially in their naïve IgM compartment. While we did isolate polyreactive clones from three IgM libraries, the only library wherein we isolated heavy chains reactive specifically to Dsg3 and VP6 was in a PV patient in remission after four rounds of rituximab, which may influence the B cell repertoire. Interestingly, all six cross-reactive heavy chains were VH1-46.

4.2.6 A subset of cross-reactive VH1-46 IgM heavy chains can both inhibit rotavirus replication and keratinocyte adhesion *in vitro.*

Upon characterizing these cross-reactive VH1-46 heavy chains more closely, we observed that all stained the surface of keratinocytes by immunofluorescence, confirming their Dsg3 specificity. Secondly, two heavy chains were able to inhibit rotavirus replication *in vitro*. Interestingly, these same two VH1-46 heavy chains inhibited keratinocyte adhesion *in vitro*. This highlights that a VH1-46 heavy chain can possess the ability to both inhibit rotavirus and Dsg3 function in the skin, pointing to the possibility

of these cross-reactive heavy chains playing pivotal roles in both rotavirus infection and in PV.

4.3 A model for the development of autoimmunity in pemphigus vulgaris

In total, there are many hurdles that are needed to be cleared in order induce an autoimmune response to Dsg3:

1.) A Dsg3-reactive B cell clone must be created
2.) This Dsg3-reactive B cell must interact with its epitope
3.) Dsg3-reactive T cells must provide help
4.) Dsg3-reactive Tregs cannot be present
5.) Tolerance must be relaxed or permissive to the differentiation of Dsg3-specific antibody secreting cells

4.3.1 A detailed description of the model

Due to reductions in E2A expression and the ability to respond to IL-7 with age, there is a lower frequency of early B cell progenitors in the bone marrow[326, 327], leading to a lower number of new B cells entering peripheral circulation. While there is no observed change in overall B cell numbers, the dearth of newly emigrant B cells in circulation results in a shift in how the B cell repertoire distributed across subsets; with a reduced contribution of naïve B cells, there is an increased representation and thus dependence on the memory B cell compartment[328, 329]. This reliance on antigen-experienced B cells may be ill-advised, as there is also a reduction in overall repertoire diversity in aged individuals compared to younger adults[330, 331].

A similar alteration in frequencies across subsets is also observed in the T cell compartment, wherein there is a reduction in output of naïve T cells due to involution of the thymus[332], and thus increased numbers of memory T cell clones within the circulating T cell repertoire[333, 334]. In addition, aged T cells are suboptimal in the ability to signal and proliferate in response to various stimuli[335], and replenishment of the T cell pool is achieved solely through peripheral T cell division[336, 337] which likely skews the diversity of the T cell repertoire.

Reduced repertoire diversity in both the B and T cell compartments of aged individuals suggests that cross-reactive clones may have an advantage in this context and thus be overrepresented, and offers an opportunity for self-reactive clones to emerge due to age-related defects in tolerance. This may in fact be the case, as levels of serum autoantibodies also increase with age[329, 338]. It has also been shown that Dsg3-reactive B cell clones can persist in PV patients after rituximab treatment[308], and is likely due to incomplete ablation of tissue-resident B cells. Perhaps these persistent clones have the ability to differentiate and secrete antibody in certain immunological contexts, ultimately leading to relapse later in life. These certain contexts could be during infection, where there may be a relaxation of tolerance in order to generate high affinity clones that can ameliorate infection. Alterations in BAFF levels could be another context[339], where it has been shown that increased BAFF levels promote the survival of autoreactive clones[340, 341]. BAFF levels have also been shown to be elevated in patients three to four months after rituximab[342, 343]. While this may be an effort of the immune system to restore the B cell population to pre-rituximab levels, perhaps within an aged individual with PV, this elevated BAFF environment may be an optimal timepoint in which to observe cross-reactive B cells. Given that we studied the IgM repertoire of only one

patient post-rituximab, further analyses with other similar patients would certainly clarify whether rituximab may be influencing the repertoire in PV patients.

It is clear from the data in Chapters 2 and 3 that there will be a B cell clone that will be Dsg3-reactive upon exiting the bone marrow simply due to V(D)J recombination. It could be singly-reactive to Dsg3; it could also cross-react to VP6 and Dsg3. With age, perhaps the declining immune system permits these self-reactive B cells to persist and acquire T cell help, and thus tolerance against self-antigens is unable to be maintained. This idea, however, requires a population of Dsg3-reactive T cells to be present, as the experiments above demonstrate that cross-reactivity in the IgG compartment is rare.

It has been shown that those that express the PV susceptibility alleles HLA-DRB*0402 and HLA-DQB*0503 demonstrate circulating Dsg3-reactive T cells[192, 207, 344]. Thus, in the populations that express these rare HLA alleles, there exists the real possibility of an autoimmune response towards Dsg3, especially considering that a single Dsg3-reactive T cell can give rise to a polyclonal B cell response in mice[210]. What differentiates a PV patient from a healthy individual in this specific population is a marked reduction of Dsg3-reactive Tregs[192, 212]. In a normal individual, there appears to be an active repression of Dsg3 Th cells by Dsg3-specific Tregs, and loss of this latter population likely results in the inability of the person to maintain tolerance to Dsg3. It remains to be known whether this Dsg3-specific Treg population is lost with age, and may contribute to the development of PV in the elderly population.

This also assumes that Dsg3 is shuttled into the secondary lymphoid organs. Given that Dsg3 is expressed in a tissue-specific manner, skin-resident Langerhans cells are the most plausible population to traffic and present Dsg3 in the secondary lymphoid

organs, as this population has been described to be essential in the immune response to cutaneous infection with *Leishmania major*[99]. While this is theoretically possible, it has never been tested, and further experiments are needed to determine whether this proves to be the case.

It is clear that simply having a Dsg3-reactive B cell, whether it be singly-reactive or cross-reactive, is merely one step in the process of developing PV. The described studies in the IgM compartment demonstrate that it is theoretically possible to create a cross-reactive BCR during V(D)J recombination. However, within the context of a rotavirus infection, these cross-reactive VH1-46 B cells likely acquire help from a VP6-reactive T cell, as a majority of the population lack of Dsg3-specific T cells. Thus, these cross-reactive B cells would likely undergo somatic mutations that increase affinity for VP6 over Dsg3. Only in a select subset of HLA-susceptible people that lack Dsg3-specific Tregs would these cross-reactive B cells acquire T cell help from Dsg3-reactive T cells, and thus become anti-Dsg3 antibody secreting cells.

Lastly, we arrived to our hypothesis that rotavirus is a potential trigger of PV due to the “shared VH gene usage theory”. Given that the phenotype does not appear to 100% penetrant in the patient population, it remains to be determined whether additional environmental triggers that can lead to PV, perhaps by inducing a different VH gene bias, or through an independent mechanism. Further studies using VirScan[345], a synthetic panel of antigens from the human virome, would allow for a high-throughput reactivity analysis of polyclonal anti-Dsg3 antibodies, and could reveal additional viral triggers that have yet to be identified using the “shared VH gene usage theory”.

4.3.2 Refuting the model

Taken together, the data above reveal that a VH1-46-biased antibody response to VP6 may give rise to a similarly biased VH1-46 autoantibody response to Dsg3 in certain individuals. While this likely represents one of several routes to developing autoimmunity, a question that remains is why there are not more cases of pemphigus vulgaris (PV) given that rotavirus is an infection that is prevalent in virtually 100% of individuals[220].

First, there is a disparity between the age at which a person would first encounter rotavirus and the typical age of onset in PV. The rotavirus vaccine is administered within the first two months of life[233], whereas the average age PV is first diagnosed is about 50-60[346]. Even in cases where the rotavirus vaccine is not administered, the penetrance of prior rotavirus infection by age five is 95%[220]. Due to this large period of time between initial encounter with rotavirus and PV, there have been no prospective epidemiological studies linking rotavirus infection with PV. Retrospective studies linking PV to any infection have also not been performed, likely due to the rarity of PV, which reduces the feasibility of these types of trials in being able to recruit enough subjects to have statistical power to observe any differences. It is also not known whether adults may experience subclinical rotavirus infection before exhibiting symptoms of PV. The fact that rotavirus testing is often not performed in the clinic[347] may also result in underreporting of the true burden of rotavirus infection in adults.

In addition, there have been no documented cases of PV arising in children after vaccination or infection. Perhaps this may be due to fully intact tolerance checkpoints in this population, which may falter in older individuals. In support of this idea, the

prevalence of autoimmunity greatly increases with age[348], which coincides with large changes in both the B and T cell compartment.

While this model is intriguing, there exists evidence of why this model may not be observed across all patients with PV. First, while we did observe at least one anti-Dsg3 IgG clone in every patient studied in Chapter 2, the utilization of VH1-46 was not predominant. Furthermore, we did not observe VH1-46 utilization across the four IgM libraries analyzed in Chapter 3; only three demonstrate an anti-Dsg3 IgM clone that utilized VH1-46, and may point to incomplete sampling of the IgM compartment. Regardless, since we cannot isolate VH1-46 clones from every patient, that alone suggests that VH1-46 biases may not be observed in every PV patient, and thus may not explain the etiology of autoimmunity for the entirety of those with PV. Further studies probing the autoantibody repertoire, such as high-throughput deep sequencing, would likely answer whether the observation of VH1-46 utilization could be observed in all patients with PV and whether non-VH1-46 Abs may be playing a larger role than I have proposed.

We also did not observe a cross-reactive phenotype across all of the VH1-46 antibodies that we did isolate from these patients. Therefore, utilization of VH1-46 is not sufficient to confer cross-reactivity. There are clearly roles for both the light chain and the heavy chain CDR3 in determining cross-reactivity. Structural studies pinpointing the key residues in binding both Dsg3 and VP6 in the cross-reactive antibodies would certainly reveal the role of the VH1-46 gene segment in cross-reactivity. The lack of cross-reactivity to VP6 suggests that this cannot be the only foreign antigen that could induce a cross-reactive response; other foreign candidates remain to be discovered. In addition, perhaps there is also a role for the polyreactive IgM clones we isolated from

three IgM libraries; polyreactivity and and cross-reactivity to foreign antigens could be two parallel avenues in the generation of anti-Dsg3 antibodies.

Alternatively, given that we did isolate Dsg3-reactive heavy chains from the IgM compartment from every patient studied points to an IgM compartment that is poised to generate anti-Dsg3 ASCs, which could be independent of the observed cross-reactive phenomenon. As I hypothesized earlier in this chapter, there may be an active suppression of these autoreactive B cells in the majority of the population, and whether they cross-react to a foreign antigen may be irrelevant, as there likely exists a population of Dsg3-reactive IgM clones in everyone. *In vivo* studies using transgenic mouse models may reveal the cellular requirements, or lack thereof, to induce an anti-Dsg3 antibody response may replicate what may occur within individuals that succumb to this disease.

What is certain from these studies is that in certain subset of individuals, VH1-46 utilization can influence the development of anti-Dsg3 autoAbs. Whether it be due to inherent reactivity solely to Dsg3, or the ability to cross-react to foreign antigens such as VP6 is not known; only future studies further characterizing this intriguing set of antibodies will reveal the true advantage of these VH1-46 Abs in the context of PV.

4.4 Future directions

In the short term, it would be fascinating to determine whether the two VH1-46 IgM heavy chains that protect against rotavirus *in vitro* would also be able to protect a mouse against rotavirus infection *in vivo.* In addition, methods to probe the B cell repertoire more thoroughly, such as deep sequencing, may reveal shared common ancestor(s) between the Dsg3- and VP6-specific VH1-46 response, as this common

ancestor may be non-reactive to both, or possess an affinity below the limit of detection that would preclude it from the selection technique we have utilized in the above studies.

Overall, the results of my thesis research have led to the support of an interesting "shared VH gene usage" theory on how autoimmune diseases arise. Due to the inherently stochastic nature of V(D)J recombination, this process confers various affinities and specificities to every B cell clone based on the genes utilized to create its BCR. The utilization of certain VH genes, for example, may increase physiological fitness in the context of binding one antigen, but not another. This has been observed in the immune response to influenza, wherein there is restricted VH1-69 gene utilization across several patients[303] due to the presence of a germline-encoded phenylalanine present in the second position of the heavy chain CDR2 that interacts with the stem of the hemagglutinin protein[304, 305].

This theory suggests that if there is an observation of shared VH gene usage in an autoAb response to a self-antigen as well as in an immune response to a foreign antigen, this may be a logical foundation on which to build a connection between these two responses. This was the basis of the second part of my thesis, which aimed to connect the VH1-46 restricted Ab responses in both the autoimmune disease pemphigus vulgaris and the common rotavirus infection, and has also been observed in the context of anti-idiotype antibodies and *Mycoplasma pneumoniae*[51, 320, 321]. However, the latter example appears to be more of a description of polyreactivity, as these autoAbs can also react to ssDNA, dsDNA, apoptotic cells[322]. In addition, it is unclear whether these anti-idiotype antibodies prevent *Mycoplasma pneumoniae* infection, which is in contrast what we observe in two cross-reactive VH1-46 antibodies that exhibit both protective and pathologic functions in the context of rotavirus and PV, respectively.

Given that my overall research interests lie in understanding how autoimmune diseases develop, a long-term goal could be to utilize this "shared VH gene usage" theory is in the context of other autoimmune disorders. It would be fascinating to determine whether the observations I have uncovered in pemphigus vulgaris could be observed in other autoimmune antibody-mediated disorders, like type I diabetes or idiopathic thrombocytopenic purpura (ITP). For example, previous studies have described an extremely restricted VH3-30 usage in the autoAbs directed against platelets in two ITP patients[294]. If we could conduct thorough VH gene usage analyses in various viral infections, and identify a particular infection that also demonstrates restricted VH3-30 usage in the Ab response to this infection, that would justify a set of studies to try and connect these two Ab responses together, similarly to what I have described in Chapter 3.

However, this theory does have its limitations. In the context of an autoimmune disease where VH gene usage is more heterogeneous, such as multiple sclerosis[349] or primary biliary cholangitis[350], proper selection of the mAbs to study would be much more difficult, as it suggests that VH gene may play less of a role in defining antigen specificity. Furthermore, in cases such as systemic lupus erythematosus, wherein patients can present with anti-nuclear Abs for up to five years before the onset of symptoms[351, 352], the role of autoAb in pathogenesis is much less clear and may not support this theory.

Broadly, an observation of shared VH gene usage across patients in response to any antigen, be it self or foreign, would suggest common epitope targeting in the antibody response to this antigen. The latter case would be extremely interesting from a vaccine perspective. For example, a better understanding of why shared VH gene usage

occurs across different patients in the context of a particular infection would likely help guide vaccine design, as it would be likely that these Abs bind similar epitopes on that foreign antigen. This theory would also imply that a similarly VH-restricted Ab response could be observed across unrelated people given a vaccine containing those epitopes, and thus upon vaccination, may observe similar protection rates in concordance with VH gene usage.

Ultimately, the studies described above provide a thorough characterization of a particular B cell response towards a self-antigen across multiple patients due to the common utilization of a particular VH gene segment, and offer a potential route for their etiologies in this human autoimmune disease model.

qPCR

cDNA was reverse transcribed from various human tissue RNA samples (Clontech) using the High Capacity RNA to cDNA kit (Applied Biosystems). qPCR studies were run and analyzed on a ViiA 7 Real-Time PCR system (Applied Biosystems) using the universal EXPRESS SYBR® GreenER™ qPCR Supermix (Thermo Fisher Scientific). Primer sets are as follows: Dsg3- 5'- TTCCTGATCACATGTCGGGC -3', 5'-CACCAGTGAGTTTGAGGCACT-3', Stro-1-5'-TTGCCAGAGCCAACGTCAAG-3' 5'-CGGCGCTGATCAGGTTGTTT-3', CD90-5'-AAGACCCCAGTCCAGATCCAG-3' 5'-TGCTGGTATTCTCATGGCGG-3', β-actin[353] 5'-AGAGCTACGAGCTGCCTGAC-3' 5'-AGCACTGTGTTGGCGTACAG-3'. Transcript abundance in each tissue sample was quantitated by the change-in-cycling threshold (ddCt) method after normalization utilizing β-actin as a control gene. Biological replicates were run in triplicate, with three technical replicates per biological replicate.

Peripheral blood collection

All patients in the above work had active PV involving both the skin and oral cavity based on clinical presentation, histology, and immunofluorescence provided by the clinic laboratories at their respective hospitals. At the time of blood draw, patients were off systemic therapies and gave consent via protocol approved by the relevant Institutional Review Board. Subsequent blood draws at various stages of disease were also acquired through the above process.

Antibody phage display (APD)

Peripheral blood mononuclear cells (PBMCs) were isolated from approximately 50 mL of blood via Ficoll (Sigma). RNA was isolated from PBMCs using the RNEasy Midi Kit (Qiagen)**.** cDNA amplification was carried out using the Superscript First Strand System (Thermo Fisher Scientific). The primers sets used to amplify all expressed heavy and light chains were generated as described[286]. Eighteen, sixteen, and six reactions were set up for amplification of lambda, kappa, and heavy chains, respectively. PCR reactions were gel electrophoresed on a 2% Agarose NuSieve® 3:1 gel (Lonza), and bands were imaged using SYBR Safe DNA Gel Stain (Thermo Fisher Scientific). Bands were purified using a Wizard SV Gel and PCR Clean-Up System (Promega) and DNA concentrations were quantitated using Low Mass DNA Ladder (Thermo Fisher Scientific). Light and heavy chains were linked together via overlap PCRs, digested with SfiI (Promega) and bulk-ligated into the pComb3x vector[286] to generate the APD library. APD libraries were electroporated into the XL1-Blue bacterial strain (Stratagene) and quantitated to determine library diversity.

To enrich for antigen-specific clones, phage were panned using ELISA. $\geq 10^{12}$ phage were bound to ELISA wells blocked with TBS- calcium (Ca) + 3% milk (Sigma) for two hours at room temperature. Wells were washed a total of ten times for five minutes each using TBS-Ca + 1% Tween-20 (Sigma), and phage were eluted with 76 mM citric acid. Phage were amplified and allowed to infect XL1 blue competent cells (Stratagene). In some experiments, the panning procedure took place at 4°C, and in other, the wash

time was reduced to two minutes. An aliquot of the phage-bacterial culture was plated onto carbenicillin-LB plates, and colonies were picked for sequence analysis and protein production. The phage-bacterial culture was amplified in the presence of M13 helper phage, and re-panned, increasing wash steps to ten. Total pans equaled four in all experiments. In cross-panning experiments, the second and fourth antigen was alternated to enrich for cross-reactive clones against both antigens of interest. Individual colonies were selected at random and analyzed for sequence.

Heterohybridoma production and screening

B cells were enriched from peripheral blood by RosetteSep (Stem Cell Technologies) using negative selection against CD2, CD3, CD16, CD56, CD66b and glycophorin A. B cell stimulation was carried out for 48 hours using either Epstein-Barr virus or inactivated Staphylococcus aureus Cowan 1 strain plus IL-2. Fusion to the HMMA 2.5 myeloma or heteromyeloma cells was carried as previously described[287, 354], and aliquoted into 96-well plates for culturing. Screening of supernatants was carried out on Dsg3 ELISA plates. Positive wells were cloned by limiting dilution, and validated for IgG as described previously[355].

Immunoglobulin subcloning, expression, and purification

F706, F779, RV6-25 and RV6-26 IgGs were cloned into the pComb3x vector for expression as recombinant single-chain variable fragments (scFv) in Top10F' cells (Thermo Fisher Scientific) transformed with plasmid. Top10F' cells transformed with

various pComb3x plasmids were shaken at 200 rpm in a 37°C incubator until an OD_{600} value of 1.0.1mM Isopropyl-β-D-thiogalactoside (Denville Scientific) was added, and the culture was shaken at 200 rpm for 6-16 hours at 30°C. Bacterial cultures were pelleted at 6000 rpm for 15 minutes, and pellets were lysed in FastBreak lysis reagent (Promega). Supernatants containing scFv were incubated with cobalt affinity Talon beads (Clontech) and rotated at room temperature for 1 hour. scFv were purified using column chromatography, and concentrated into PBS. Protein concentrations were validated with NanoDrop (Thermo Fisher Scientific) and Coomassie gels.

Sequence analyses

To determine somatic mutations and V(D)J segments utilized by each antibody, sequences were run through IMGT/V-QUEST[27, 356], Vbase2[357], and in some cases, SoDA[358]. Statistical analyses using the BASELINe test[290, 291] utilized differences in expected versus observed somatic mutation frequencies as a read out for positive or negative selection. Expected mutations were calculated based on somatic mutation hot spots and substitution biases within each monoclonal Ab (mAbs). P values<0.05 were considered significant.

Antibody mutation experiments

Somatic mutations identified in each mAb were reverted to the corresponding germline nucleotide based on IMGT/V-QUEST, Vbase2, and in some cases, SoDA. In cases where there was more than one GL heavy chain (HC) CDR3 identified, both were

synthesized. GL light chain (LC) and HCs were synthesized as minigenes (Integrated DNA Technologies). The following primer sets were used both amplify and append SfiI sites to the 5' and 3' ends of the LC: LC- 5'-GGGCCCAGGCGGCCGAGCTC-3' and those listed in Cho et al. The HCs were amplified in a two step-process. The primer sets are as following: 1st round: 5'-GGTGGTTCCTCTAGATCTTCCTCCTCTGGTGGCGGTGGCTCGGGCGGTGGTGGGCAGGTGCAGCTGGTGCAGTCTGG-3', 5'-ACGCGCACAGTAATACACGGCCGTGTC-3', 2nd round: 5'-GACACGGCCGTGT ATTACTGTGC-3', 5'- -GGGCCGGCCTGGCCACTAGTGACCGATGGGCCCTTGGTGGAAGCTGAGGAGACGGTGACC-3'. The LC and HC were amplified and overlapped using Platinum Pfx DNA polymerase (Thermo Fisher Scientific). Overlapped LC and HCs were ligated into the pComb3x vector using the Gibson Assembly Cloning Kit (New England Biolabs). For some GL mAbs, the entire sequence was synthesized as a geneBlock (Thermo Fisher Scientific), amplified via Platinum Pfx DNA polymerase (Thermo Fisher Scientific) with the following primer set: 5'- GGGCCCAGGCGGCCGAGCTC-3', 5'-GGGCCGGCCTGGCCACTAGTGACCGATGGGCCCTTGGTGGAAGCTGAGGAGACGGTGACC-3' and digested with SfiI (Roche). Digested DNA was purified using the Wizard SV Gel and PCR Clean-Up System (Promega) and ligated into the pComb3x vector using T4 DNA ligase (Thermo Fisher Scientific). Point mutations in various mAbs were carried out using the Quikchange Lightning Multi Site-Directed Mutagenesis Kit (Agilent).

Enzyme-linked Immunosorbent Assays (ELISAs)

Dsg3 (MBL International, Euroimmun) and Hep2 (IBL International) ELISAs were performed according to manufacturers' directions, except in cases where mAb binding took place at 4°C overnight. Some mAbs were tested as unpurified bacterial lysates in cases of inefficient production. To make VP6 and BSA ELISA plates, 5 ug/mL of each protein (DLPs or purified BSA) was diluted in PBS-Ca (Thermo Fisher Scientific) and incubated in 96 well plates (Corning) at 4° overnight. VP6 plates are made using rotavirus DLPs. Plates were washed with TBS-Ca with 1% Tween-20 (Biorad) before blocking with TBS-Ca + 3% milk (Biorad, Sigma) for 1 hr at room temperature. VP6/BSA ELISAs were then carried out according to the standard protocol. In some cases, plates were developed with high sensitivity TMB (Biolegend, Thermo Fisher Scientific). Cutoff values were determined by calculating three standard deviations above the mean of the negative controls.

Immunofluorescence (IF) microscopy

Hep2 IF slides (Euroimmun) were developed according to manufacturer's directions. Normal human skin was acquired through the Penn Skin Disease Research Center (SDRC), and sectioned onto glass slides (Thermo Fisher Scientific). Monkey esophagus slides were purchased from SciMedX. Slides were blocked with TBS-Ca +1% BSA (Biorad, Sigma) at room temperature for 30 minutes. Slides were washed with TBS-Ca three times, and incubated with scFv diluted in TBS-Ca +1% BSA at room temperature for 1 hour. Slides were washed as above, and incubated with 1:100 rat anti-HA Ab (Roche) diluted in TBS-Ca + 1% BSA for 1 hr at RT. Slides were washed as

above, and incubated with 1:200 Alexa Fluor 594 donkey anti-rat IgG mAb (Thermo Fisher Scientific) for 30 minutes at room temperature. Slides were washed as above, fixed with 95% ethanol, and mounted with mounting medium (KBL). Immunofluorescence was visualized with an Olympus BX61 microscope and images acquired using Slidebook 4.2 software (Olympus) and a Hamamatsu Orca ER camera.

Pathogenicity assays

Normal human skin biopsies obtained by the Penn SDRC were injected with 50 ug of mAb at the dermal-epidermal junction, and incubated in defined keratinocyte (DK)-SFM media supplemented with 1.2mM calcium (Thermo Fisher Scientific). 0.8 µg of exfoliative toxin A (Toxin Technology) was added as necessary to injection mixtures. After an overnight incubation at 37°C, skin samples were subjected to a Nikolsky test, and bisected. Half was embedded in phosphate-buffered formalin (Sigma), and the other embedded in OCT (Electron Microscopy Sciences) for histology or IF, respectively.

Human primary keratinocytes were derived from the Penn SDRC from neonatal foreskin using Institutional Review Board-reviewed protocols and seeded into 12-well tissue culture plates with DK-SFM media (Thermo Fisher Scientific). Confluent wells were incubated with mAbs diluted in DK-SFM media supplemented with 1.2 mM calcium for 6 hours at 37°C. 100 ng exfoliative toxin A (Toxin Techonology) was added as necessary to mAb mixtures during the last 2 hours of the incubation. Wells were washed with PBS-Ca and incubated with dispase (Roche) at 37° C for 30 minutes. Cell monolayers were washed with PBS-Ca and subjected to 5 rounds of pipetting with a 1

mL pipettor (Rainin). Cell monolayers were stained with Crystal Violet (Sigma), and cell fragments were counted using Image J. Cutoff values were determined by calculating three standard deviations above the mean of the negative controls.

Epitope mapping

300 uL of baculoviral supernatant containing Dsg1 molecules with domain-swapped Dsg3 EC domains[359] (gift of Dr. Masayuki Amagai) were incubated with mAb for 30 minutes at RT. mAbs were immunoprecipitated with anti-HA agarose (Sigma) overnight at 4°C. Reactions were run on SDS-PAGE gels (Biorad) and transferred to nitrocellulose (Biorad) for development with anti-E tag-horseradish peroxidase antibody (GE Healthcare) via Western blot.

Surface plasmon resonance

Mouse anti-human IgG mAb (Biacore) was amine-coupled to a CM5 biosensor chip using N-hydroxysuccinimide/N-ethyl-N0-[3-(dimethylamino) propyl]carbodiimine hydrochloride (Pierce). The chip was blocked with 1M ethanolamine, pH 8.5 for 7 minutes. Baculoviral Dsg3-F_c protein was flowed over one cell; a second cell was used as a reference cell and did not contain Dsg3-F_c. Assays took place at 25° in HBS-EP buffer (Biacore). Each mAb was run at least three times over several concentrations. To adjust for nonspecific background, the reference cell was used to subtract changes in refractive index leading to a bulk shift from the overall mAb binding signal in each condition. After each condition, the chip was regenerated using 3M $MgCl_2$. An average of

500 response units was reproducibly achieved for Dsg3-F_c binding to the biosensor chip across all replicates. All data were fit to 1:1 Langmuir binding model and analyzed with the BIAevaluation 3.0 software (Biacore). Any mAbs with χ^2 values greater than 2 or considerable bulk change added to the model were also tested using the heterogeneous ligand or conformational change models.

Rotavirus spreading infection assay

Lipofectin (Thermo Fisher Scientific) was incubated 15% v/v with serum-free EMEM media (ATCC) at RT for thirty minutes. Rotavirus DLPs were added to the lipofectin-treated media at 2 µg/mL and incubated for 40 minutes at RT. Abs were diluted 1:4 starting at 25 µg/mL in serum-free EMEM, added 1:1 to the DLP-lipofectin mixture, and incubated at 37°C for 1 hour. 40uL of DLP-Ab mixture was added to 100% confluent monkey renal MA104 cells (ATCC) in a 96-well plate (Corning) and incubated at 37°C for four hours. 40 µL of 20% fetal bovine serum (FBS) and 2% penicillin streptomycin (Thermo Fisher Scientific) was added to wells and incubated at 37°C overnight.

Cells were washed with PBS-Ca (Thermo Fisher Scientific) and fixed with ice-cold methanol for 15 minutes at -20°C. 50 µL of 1:500 polyclonal goat anti-rotavirus Ab (Fitzgerald) was added and incubated at 37°C for 1 hour. Cells were washed with PBS-Ca and incubated with 50 µL donkey anti-Goat-HRP secondary antibody (Abcam) at 1:1000 for 1hour at 37°C. Cells were washed with PBS-Ca and stained with the Pierce DAB Substrate Kit (Thermo Fisher Scientific). Cells were washed with PBS-Ca and wells were imaged on an EVOS FL Auto Cell Imaging System (Thermo Fisher Scientific).

Rotavirus foci were quantitated using ImageJ and the Immunohistochemistry Image Analysis Toolbox. Percent reduction was calculated based on the difference in number of foci in each Ab condition compared to the no antibody control. Antibody conditions were run in duplicate and imaged at least once per well. Error bars indicate SEM. Cutoff values were determined by calculating three standard deviations above the mean of the negative controls.

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