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B VIRUS NEUTRALIZATION AND SPECIFIC ANTIBODY INDUCTION: EACH
DEPENDENT ON UNIQUE GLYCOPROTEIN B EPITOPES.

by

VISHAKH WALIA

Under the Direction of Julia K. Hilliard, Ph.D.

ABSTRACT

B virus (BV, *Macacine alphaherpesvirus 1*), a neurotropic Simplexvirus, is enzootic in macaque monkeys, infects all of the 17 species of the macaque monkeys including *Macaca mulatta*, *M. fascicularis*, and *M. nemestrina*, the most extensively used nonhuman primate biomedical research models, but lifelong, mostly latent, zoonotic infection in humans often leads to severe neurologic impairment or fatal encephalomyelitis in up to 80% of reported cases. Early detection is key as no virus-eliminating therapeutics are available currently. Antivirals are available as a prophylactic measure, or as an effective therapy only if administered very early in acute, symptomatic infection. Because virus shedding is rare, serological detection of BV-specific antibodies is often the only diagnostic option for identifying BV infection. Current BV

diagnostic methods are labor-intensive complex assays and even these must be performed frequently to differentiate cross-reactive BV and HSV antibodies. Hence, it is critical to identify markers of specific immune responses and virus neutralizing targets. To define the relevance of BV gB in controlling the BV infection, by accurate diagnosis and informing rational vaccine design, the hypothesis “B virus neutralization and specific antibody induction are each dependent on unique glycoprotein B epitopes” was tested.

Epitope mapping of the neutralizing mAb identified novel mimotopes, corresponding to aa residues 115-120 and aa residues 515-525 of BVgB, that are potential targets for virus neutralization and may serve as epitope-focused recombinant protein-based BV vaccine candidates. Epitope mapping of the non-neutralizing mAb, using phage display and peptide array, revealed a B virus specific epitope that can identify anti-BV polyclonal antibodies from both BV infected humans and macaques without cross reacting with HSV1 and 2 polyclonal antibodies. Through this study I propose a novel, simple, rapid and cost-effective method of fine mapping linear epitopes using phage display and compound mutagenesis that circumvents the use of expensive peptide array and laborious mutagenesis scanning techniques. The impact of this study will inform vaccine designs that will block virus entry, as well as the development of novel strategies for early and unequivocal identification of B virus zoonotic infections in individuals with highly cross-reactive antibodies.

INDEX WORDS: Herpesvirus, Glycoprotein B, Epitope mapping, Phage display, Peptide array, Differential diagnostics, Fusion, Monoclonal antibodies, Vaccine

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A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2020

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August 2020

DEDICATION

To my parents who have been a source of constant support and motivation. The valuable life lessons learned from you have helped me in my journey and continue to do so. You have helped me strive and prevail through every adverse situation. All the values that you have instilled in me have always motivated me to do the right thing and to do it right. I am truly blessed to have you as my parents.

To my grandpa, you are my role model. I have learned self-discipline from you. You have motivated me to stick to my principles even in the toughest of situations. Thank you for making me look at the bigger picture, boosting my morale, and cheering me up whenever I was down. Talking to you and remembering grandma keeps me grounded.

To my elder brother Anshul who has always been by my side. Despite having to deal with the challenging situations in your own life, you have always supported me and helped me in numerous ways so that I could be where I am today. I feel proud to have a brother like you.

To my fiancé Audrey for being so strong, patient and open with me. Thank you for having faith in me, loving me for who I am, encouraging me whenever I felt low, and helping me manage my stress. I feel so lucky to have you and your wonderful family in my life. I can't wait to start our lives together.

To my friends back home in India and here in the US. Shrikant, my best friend in this foreign land, we have been through some crazy experiences, it's been a hell of a ride. I will always cherish our memories. Joshua, you are a true leader, and I appreciate all your support and help with BGSA and Ga-Bio during my time at GSU. My school and college buddies Sukrit, Abhinav, and Santosh, you all have been an essential part of my support system. Thank you for talking to me from time to time, encouraging me and supporting me.

ACKNOWLEDGMENTS

First and foremost, I would like to thank Dr. Hilliard for believing in me and giving me the opportunity to be part of the B virus lab family. Your teachings on avoiding bias in science, the importance of effective communication of science, and the impact of the written word have helped me become a responsible scientist and a better version of myself. Thank you for providing a truly conducive research environment, being patient with me, for being understanding and appreciative, and challenging me. Thank you for allowing me to take time to grow outside the lab via extracurricular classes and work.

To Dr. Katz, my other advisor, this work would not have been possible without you. Thank you for helping me learn all the techniques and discussing data with me on a day to day basis, the several brainstorming sessions, helping me stay positive, motivating to believe in myself, and inspiring me to stay persistent.

My committee members Dr. Dix and Dr. Houghton, for leading me in the right direction and keeping me focused. Thank you for providing valuable inputs and suggestions and constructive criticisms.

To Dr. Irina Patrusheva, thank you for all your guidance and the troubleshooting sessions. Thank you for sharing your practical knowledge and experience, they have been critical for the success of multiple experiments.

To Nina Beato and other diagnostic staff, you all are amazing, your patient records keeping and organizing skills have helped me tremendously.

To all my wonderful current and previous lab mates, I have truly enjoyed our time together. Thank you for making this journey rich of memories and experiences that I will cherish forever. I wish you all the best in all your endeavors.

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LIST OF ABBREVIATIONS

gB	Glycoprotein B
aa	amino acids
NGS	Next generation sequencing
sulpho-NHS	Sulfo-Nhydroxysuccinimide
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
TBS	Tris-buffered saline
TBST	TBS containing 0. 5% Tween-20
BBST	Borate Buffer Saline 0. 5% Tween-20
2xYT	Yeast Extract Tryptone Bacterial growth media
LB	Luria Bertani
IPTG	isopropyl- β -D-thiogalactoside
Xgal	5-Bromo-4- chloro-3-indolyl- β -D-galactoside
RT	Room temperature
mL	milliliter
μ g	microgram
μ L	microliter
Min	Minutes
O/N	Overnight

1 INTRODUCTION

1.1 B Virus

Herpes B virus (BV), endemic in macaques monkeys, is a member of family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Simplexvirus* [1-3]. The linear DNA genome of ~155 kbp with about 75% G+C content is enclosed in an icosahedral capsid present in an unstructured tegument protein surrounded by a lipid envelope embedded with 12 glycoproteins [4, 5].

Its natural hosts include all the 17 macaque species. The rhesus and pigtail macaque BV strains have a genome identity >99% while the genome identity amongst other macaque BV strains is ~89–95%. It is genomically and immunologically related to herpes simplex virus types 1 and 2 and other closely related monkey alpha herpesviruses such as Baboon Herpesvirus Papio 2 (HVP-2), herpesvirus simian agent 8 (SA8) and chimpanzee herpes virus (ChHV) and to a lesser extent the viruses of squirrel monkeys (HVS1) and spider monkeys (HVA1) [6-13].

The BV is transmitted horizontally via direct contact and exchange of bodily secretions [14-16]. BV infections in humans although rare, but often leads to severe neurologic impairment or fatal encephalomyelitis in 70-80% cases. B virus infects mucosal epithelia, the epithelial and dermal layers of the skin, from there it spreads to the sensory neuron endings of the sensory ganglia, travelling retrograde, it ultimately spreads to the central nervous system (CNS). The survivors are left with lifelong neurological disorders and associated problems. Majority of the transmission occurs due to contact with captive macaques via bites or scratches but other modes of transmissions are contact of mucosal membranes with infected macaque urine, feces, or cultured cells or via needlestick injury [17-19]. So far there has been only one case of a human to human transmission [20].

BV is considered a macaque equivalent of human HSV, as just like HSV its rapid lytic replication cycle produces extracellular progeny after about 6 hours of infection and the BV proteins are synthesized as a part of immediate early/early/late gene expression program [21]. Moreover, the BV genetic arrangement is orthologous to that of HSV and it rarely causes fatal infections in its natural host -macaques. BV glycoproteins gC (UL44), gD (US6) and VHS (UL41) have shown to have structural and functional properties similar to HSV but such similarities have not been studied for other proteins.

1.1.1 B virus entry

Generally, for the entry of an enveloped virus a single surface glycoprotein binds to its receptor on a host cell membrane and brings the viral envelope and the cell membrane in close proximity to initiate fusion. But in herpes viruses this function is performed by three glycoproteins gB and gH/gL in conjunction with a fourth accessory protein. Although, different herpesviruses may utilize different accessory proteins, gD (HSV), gp50 (Porcine herpes virus; PRV), UL128, UL130, UL131 (HCMV), gp42 (EBV), for viral entry but the “core fusion machinery” is formed by three surface glycoproteins gB and gH/gL heterodimer, whose functions are conserved across the herpesviruses, are involved in fusion [22-31]. The structure of gB is conserved across all herpesviruses whereas gH/gL structure varies.

The accessory proteins, for e.g. gD in case of HSV, are required for cell tropism or to initiate the process of virus entry. The binding of gD to its receptors releases gD from its autoinhibited state that triggers a conformational change in gH/gL and subsequently gB that exposes the hydrophobic fusion domains of gB responsible for virus– cell and cell– cell fusion. The gD receptors are not conserved across the herpes viruses. The HSV gD interacts with

HVEM and nectin-1 on the host cells to activate the core fusion machinery but pseudorabies virus (PRV) can only use nectin-1 as a gD receptor but cannot use HVEM. Similarly, BV gD utilizes Nectin-1 but not HVEM or PILR α for cell-cell fusion and virus entry [32, 33]. But BV can utilize both gD dependent and independent cell entry and transmission mechanisms, hence gD might be dispensable for virus entry [34].

BV gH forms the larger part, 847 aa residues, of the gH/gL complex whereas gL forms the smaller part, 224 aa residues, of the complex. Herpesvirus gH are generally composed of three structural domains, a N-terminal domain that is considered unstable alone but is stabilized by gL, a central helical domain and a C-terminal β -sandwich domain. The latter two domains are structurally conserved across all the herpes viruses. These domains contain heptad-repeat and fusion peptides that are mostly buried in the core of the protein. The gH/gL complex was believed to be a fusion protein due to the presence of the fusion peptides, but as the crystal structure was solved no resemblance to any known viral fusogen were found [35-37]. Moreover, fusion cannot occur without an interaction between gH/gL with gB[38]. Multiple studies have shown that gB is the only fusogen of HSV used in virus entry, and gD and gH/gL are only the regulators of this process [37, 39, 40].

1.1.2 B virus glycoprotein B

BV glycoprotein B is composed of about 891 aa residues with aa residues 98-712 forming the ectodomain, aa residues 713-761, 762-783 and 784-891 forming the predicted membrane proximal region, transmembrane domain and cytoplasmic domain. In addition to being the one of the most immunogenic BV glycoproteins it is also postulated to be the BV fusion protein as the BV neutralizing mAbs used in this study have been found specific to gB

[41-43]. The crystal structure of BV gB has not been solved but since the gB structure is highly conserved across the herpes viruses Herpes simplex virus glycoprotein B will be used as a reference throughout this study. The herpes virus gB is a homotrimer that has been classified as class III viral fusion protein, possessing the features of both class I and class II fusogens [44, 45]. The herpes virus gB, like other class III fusion proteins contains short hydrophobic segments at the base of the protein that help the virus to form pores in the host cell membrane.

Class I fusogens are trimeric with their fusion loops hidden internally in the pre-fusion state. The post-fusion state is different from prefusion and is characterized by group of six α -helices [46]. Class II fusogens are also trimeric but are made up of multiple β -pleated sheets that can be arranged as either homodimers or heterodimers with internally located fusion domains [47]. Class III fusogens, like class I and II are trimeric but contain both α -helices and β -pleated sheets. They dissociate during fusion and return to a different post-fusion state that is different from pre-fusion state [45, 48].

The fusion loops appear as multiple short hydrophobic segments of aa that form a ridge surrounded by charged residues. HSV1 has two fusion loops found on the domain I, as described by Heldwein et al. fusion loop 1 (FL1; region aa 173 to 179) and fusion loop 2 (FL2 258 to 265) [44, 49]. But neutralization of the virus is not solely reliant on blocking of the fusion loops. Multiple neutralizing mAbs have been shown to bind on different domains of gB besides the fusion loops [50].

The fusion process is regulated by the membrane proximal region (MPR) of gB. The MPR covers up the fusion loops present in the domain I of gB [51]. About 40% residues of MPR are highly conserved herpesviruses and mutation of these residues renders virus non-infectious

[52]. The gB mediated fusion is also regulated by gH ectodomain and a short gH cytotail. The mutation of more than 6 aa residues of the gH cytotail impairs the fusion mechanism [53]

Hence by mapping the gB epitopes important vaccine candidates and other markers of diagnostic value that can be used to identify BV specific immune responses can be discovered.

1.1.3 Threat by *B virus*

Fewer than two-dozen pathogens cause more than five million deaths each year. Over 300 million humans a year become sick every year with over half the world's population being at risk on any given day. The critical barrier to control infectious diseases including new and recurring emerging infections is largely our lack of knowledge about which immune defenses are effective at limiting pathogen spread. B virus, *Macacine alphaherpesvirus 1*, is one such virus that often causes a fatal zoonotic disease that has to be controlled. This infection costs funding organizations globally many millions of dollars annually, yet these resources only minimize the ever-present threat of zoonotic infection.

Three of the 17 species of macaque monkeys that comprise the genus *Macaca* species *M. mulatta*, *M. fascicularis*, and *M. nemestrina* have been used extensively as biomedical research models for nearly a century making BV the most serious health occupational hazard for people working with macaques. [1, 2, 54]. Individuals who become infected via bite, scratch, splash, or fomites caused by the natural host, members of the genus *Macaca sp.*, remain infected for life. They may survive as a result of timely antiviral intervention. The mortality in untreated cases is about 80%. Survivors may shed periodically after recovery [55-58].

1.1.4 Diagnosis of *B virus*

Due to the neurotropic nature of BV, and establishment of latency in the peripheral neurons, and the unpredictable viral shedding, the definitive diagnosis using virus culture or

virus-specific nucleic acids detection (PCR) is impractical. Although PCR assays may not be useful in case of latent infections, they are a robust method of detecting miniscule amounts of viral DNA in swabs from bites or scratch. The infected individual and the captive monkey responsible for the infection can both be tested for a more definite diagnosis. Several PCR methods are being currently used [63, 64]. Since an individual or a monkey could be infected with different strains, or multiple strains simultaneously, of BV diagnosis using PCR might become challenging due to significant sequence differences amongst the virus strains [65-68].

Hence serology is the only practical option. But concurrent infections with HSV1 and 2 further complicates the serological diagnosis of B virus due to the presence of cross reactive HSV1 and HSV2 antibodies extensive antigenic cross-reactivity between BV and HSV [7, 8, 60-62]. The current B virus diagnostic methods such as titration ELISA (tELISA) and the confirmatory tests such as western blot analysis, the recombinant-based ELISA, and competition ELISA that cannot always distinguish between BV and HSV antibodies need to be updated [41, 42, 62].

To develop simple and accurate serological differential diagnostic tools it is therefore critical to identify B virus specific epitopes that can distinguish between BV specific and HSV1 and HSV2 specific antibodies. To this end, our laboratory has identified and characterized a panel of B virus specific mAbs [43]. One of such BV virus specific mAb 9F1, that does not cross-react with HSV1 and HSV2, will be used to map BV specific functional epitopes using a unique approach that uses random peptide phage display, overlapping peptide array and compound mutagenesis scanning.

1.1.5 Interventions against B virus

In addition to having a high fatality rate, the B virus infections just like herpes simplex virus infections persists for the lifetime of the host. The appearance of symptoms can vary greatly between individuals can be managed by using antivirals such as acyclovir, valacyclovir, and famciclovir, but they may only work for mild symptoms and not for severe symptoms that can appear as early as 8 days[16, 57, 69]. Also, it is not practical to provide these drugs as prophylaxis to the individuals that might be considered at high exposure risks, for e.g. primate workers. Moreover, BV has found to be less sensitive to these drugs, in some cases as much as 10 folds less effective than HSV [70-72]. Other drugs like penciclovir (PCV), ganciclovir (GCV), cidofovir (CDV), and an experimental drug FEAU provide better protection [71, 72]

Hence, it is vital to have a vaccine that can control the viral infection. The vaccine might be the safest prophylactic measure that can be administered to the individuals at high risk of infections. Hence, in an attempt to identify the vaccine candidates, specific targets of the B virus neutralizing antibodies must be identified. In this study we will attempt to identify the targets of neutralizing anti-B virus gB monoclonal antibodies using the phage display method.

Another manifestation of the BV targets identified by this study could be to use the markers in combination with a non-toxic topical viricidal drug that can either effectively block virus entry or precipitate the virus at the site of entry. This could be an effective way of providing rapid prophylactic treatment prevent the virus from entering and replicating.

1.2 Monoclonal antibody epitope mapping

Antibodies or in other words immunoglobulins (Igs) are one of the most versatile and robust components of the immune system of all vertebrates. Their ability to specifically identify antigenic determinants makes them an important tool for diagnostics and immunotherapeutics.

Understanding the antibody- epitope interactions is the first step towards developing an effective diagnostic and epitope-focused recombinant protein-based BV vaccine. To this end we will be using three monoclonal antibodies, produced by immunization of mice with BV Ag, one BV type specific and two neutralizing to map epitopes responsible for inducing specific antibodies and virus neutralization to discover epitopes responsible [43]. Following approaches will be used in this study for epitope mapping:

1.2.1 Random peptide Phage Display libraries

Phage display is a selection method in which a heterologous short segment of a protein is fused with a capsid protein of a bacteriophage and displayed on the virion surface. It was first described by George P. Smith in 1985 when he used phage gene III of M13 bacteriophage to create a fusion protein [73]. The recombinant bacteriophages thus produced could be enriched and provided a physical link between the expressed peptide and the encoding DNA. Peptides of variable lengths 6-12 aa residues in length, that may or may not be di-sulphide restricted, are usually expressed on the bacteriophage and are used to map linear and/or conformational epitopes of a target. Each peptide expressed is presented on the pIII coat protein or the pVIII coat protein. When the peptide is fused to pIII five copies of the peptides are displayed per phage on each of the pIII coat protein. When the peptides are fused to pVIII gene several hundred copies of the peptide are expressed on each of the pVIII coat protein. Although, if all the pVIII coat proteins are fused with a peptide it may compromise the function of the protein and ultimately the phage viability. Hence to circumvent this issue hybrid phages are produced in which some of the pVIII coat proteins are wild type and some are fused to the peptide. Alternatively, a phage hybrid can be created using a phagemids. In this method a bacterial host is co-infected with a

phagemid that carries the genes encoding the fusion protein and a helper phage carrying the genes required for the formation of phage particles.

Peptides specific for the target antibody(s) are identified by affinity selection (aka biopanning). For biopanning the phage library is incubated with the target to allowing the phages expressing peptides, that represent either the complete, partial epitope or a mimotope, to bind to the target antibody. The unbound phages are washed away, and the bound phages are eluted. The eluted phages are titered and amplified. This completes one round of positive selection. A round of negative selection is performed using a non-specific target, to minimize the selection of any non-specific phages, and the unbound phages are amplified. These amplified phages should undergo two more rounds of positive selection to enrich the phage population specific for the target. The phage population thus obtained is sequenced.

Phage display (PhD) has several applications besides mAb (B cell receptor) and T cell receptor epitope mapping such as discovering the target molecule of a cell receptor. The cell membrane receptors are key players in cell signaling functions and hence are targets of potential drugs [74, 75]. Protein-protein interactions play a major role in governing the normal physiological functions. PhD has been used to find partners of highly conserved protein domains such as Src homology (SH) 3 domains [76]. Organ specific peptides can be identified using PhD. This method was used to discover brain vasculature targeting peptides [77]. To improve the therapeutic index and reduce the emergence of drug resistance anti-cancer drugs have been delivered to solid tumors using peptide mediated liposomes[78]. The peptide mediated liposome is constituted of an anticancer drug, a liposome and a targeting ligand (peptide). To discover the target ligand phage display is used.

1.2.2 Linear peptide array

This method is a derivative of the method first described by Mario Geysen in 1984 known as Pepscan. A library of peptides, usually the entire sequence of a particular protein is coated on a surface as overlapping peptide and the desired antibody is used to scan the coated peptides to locate its target [79]. A positive reaction produces a fluorescent signal which is read by an optical reader. If two distant peptides bind to the same target of interest it signifies that the two peptides are parts of a discontinuous epitope. This technique allows the detection and quantification of simultaneous multiple binding reactions from miniscule amounts of sample. This technique can only be used only to identify the linear epitopes of a target. Moreover since the plate is coated with linear peptides only any antibody specific to any of the post translational protein modifications such as acetylation, methylation, phosphorylation, glycosylation will not bind to the coated peptides.

1.3 Summary

In summary, BV infection in humans either causes a lifelong severe disease or results in death. To tackle the disease caused by B virus there is an urgent need to develop effective tools for unequivocal diagnosis of B virus. Since BV glycoprotein B is one of the most immunogenic glycoproteins it must be used as a prime candidate for the discovery of specific markers that can distinguish B virus specific antibodies. To control the virus spread of the virus in infected individuals again B virus gB should be explored for any markers responsible for neutralization of the virus as gB is most likely the only fusogenic glycoprotein out of all the 12 BV glycoproteins. By targeting the gB epitopes responsible for the fusion the virus spread and thus the infection can be controlled. Hence the goal of this study is to identify the role of a specific glycoprotein, gB, in neutralization of the virus and induction of type-specific antibodies. To accomplish this goal we

have two objectives: 1) identify epitope(s) that induces (a) neutralizing, and (b) type specific antibodies, and 2) exploit this new information to inform rational vaccine strategies to block B virus infection, as well as to ensure rapid and unequivocal identification of B virus infections. To accomplish these objectives, the hypothesis that **B virus neutralization and specific antibody induction are each dependent on unique Glycoprotein B (gB) epitopes** should be tested. To test this hypothesis, the following sub-hypotheses presented in each specific aim will be tested.

Aim 1. B virus gB epitope corresponding to alpha herpesvirus fusion loop domain is a target for B virus neutralization.

Aim 2. Specific gB epitope(s) induce unique, B virus-specific humoral response(s) differentiable from those induced by closely related members of the virus subfamily.

2 AIM 1: TEST IF B VIRUS gB EPITOPE CORRESPONDING TO ALPHA HERPESVIRUS FUSION LOOP DOMAIN IS A TARGET FOR B VIRUS NEUTRALIZATION.

Untreated B virus infections results in mortality in 80% of the cases. Since no therapeutics are currently available against B virus it is critical to identify vaccine candidates that could be used to treat the B virus infections. Preliminary plaque reduction neutralization assays (PRNT) using the B virus neutralizing antibodies IgG1 5A2 and 4E11 have shown that these neutralizing antibodies are able to neutralize the BV, SA8 and HVP2 viruses up to 75%, 63% and 33%, respectively and target BV gB. The PRNT and the western blot results show that the two monoclonals have a similar pattern of functional activity and are thus closely related. Out of the 12 glycoproteins present on the envelop of the B virus gB, gC, gD and mgG have found to be the most immunogenic [2, 80]. Several neutralizing antibodies have been mapped to the fusion

loops of the glycoprotein B (gB), a class III fusion protein, of HSV1 [81-83]. The binding of neutralizing antibodies to the fusion loop has shown to block the cell-cell fusion [84]. Hence establishing the fusion loops as the targets for neutralization in HSV1 infections. Since the crystal structure of BV has not been solved yet, the question arises if BV gB, just like HSV1 gB, is also a class III fusion protein that is required for fusion, and therefore is an important target for neutralization of the B virus just like HSV1.

The role of gB and gH/gL in herpes virus fusion has been well characterized in HSV1 [38, 85, 86]. The entry mechanism of Herpes simplex viruses is believed to be conserved across species and generally requires four membrane glycoproteins: gD, gB and heterodimeric gH/gL. gD is the receptor binding protein that defines the cell tropism, gB cannot cause fusion on its own and gH/gL is another glycoprotein required for fusion[87, 88]. Hence, the fusion of BV might be dependent mostly on gB and gH/gL. The blocking of gB's interaction with gH/gL blocks fusion in HSV1 infections[38, 85]. Hence, we postulate that the neutralizing antibodies against gB will either bind to the gB fusion loops directly or to the gB motifs that interact with gH/gL and abrogate the interaction of gB and gH/gL and thus inhibit the entry of BV. Thus, identifying the epitopes of BV neutralizing mAbs using phage display libraries and mapping their location on a BV gB model, based on the BV sequence and crystal structure of HSV1 gB, will help us identify the motifs involved in viral neutralization and their role in viral entry. The peptides synthesized based on the epitope sequences will represent the immunogenic regions of the gB that are important for inducing neutralizing antibodies against B virus in the host, hence the active peptides will serve as an important vaccine candidates.

2.1 Identify the epitope/mimotope sequence(s) recognized by BV neutralizing mAb 4E11 by random peptide phage display library.

To identify the epitopes of gB that might be involved in the fusion machinery of the B virus the epitope mapping of the BV neutralizing mAb IgG1 4E11 using commercial random peptide 7-mer (heptapeptide), 12-mer (dodecapeptide) and cyclic (loop-constrained heptapeptide) phage libraries were employed. The twenty phage clones obtained after a round of positive phage screening followed by a round of negative screening and then two consecutive rounds of positive selection phage screening from each library were tested for their specificity for the mAb 4E11 in a phage ELISA (Fig. 1). Out of the twenty clones picked from each of the three libraries only one phage clone- Clone 20 from the Cyc phage library bound to the mAb 4E11 with an OD (A_{450}) of 0.773. The clone 20 did not bind with the anti- BV VP13/14 mAb 12F5C1, hence exhibiting that it was specific to the 4E11. None of the other clones, including ones selected from other libraries, bound to either of the mAbs. This shows that there was a poor enrichment of the 4E11 specific phages by the third round of the screening.

To confirm that this phage clone was specific for 4E11 and to test if it was also specific to the closely related 5A2 mAb the phage clone was directly coated on an ELISA plate and tested for its ability to capture the mAbs 4E11, 5A2 and 12F5C1 (Fig. 2). The clone 20 bound to both mAb 5A2 and 4E11 and not to 12F5C1. The binding of the clone 20 with 5A2 increased at a higher rate compared to 4E11 with the increasing concentration of the mAbs (Fig. 2a). The binding of 5A2 to the clone was significantly higher than the binding of 4E11 ($p < 0.005$) and 12F5C1 ($p < 0.0001$) to the same clone at the dilution of 40 $\mu\text{g/mL}$ of the mAbs. These results show that the mAb 5A2 is more specific to the phage clone identified by the mAb 4E11 than the 4E11 itself. The clone 20 was sequenced by sanger's method. The DNA sequence was translated and

aligned with the conserved phage sequence to reveal the amino acid sequence CEPFWTNCGGGS.

2.2 Identify the epitope/mimotope sequence(s) recognized by BV neutralizing mAb 5A2 by random peptide fd (landscape) phage display library.

The commercial phage display libraries used in the previous experiment were limited in the types of peptides (7,12-mer and cyclic) and number of peptides expressed per phage. The phage libraries used earlier comprised of phages expressing only 7-mer and 12-mer linear and cyclic 7-mer peptides on each of the five pIII proteins of the phages. Both of these issues were resolved by using a fd phage (landscape) library that comprised of phages expressing either one of the linear or looped 8,6,10,12-mer peptides on about half of the hundreds of the pVIII proteins of the phages. Moreover, since the mAb 4E11 wasn't highly specific for the selected phage clone in the previous experiment (Fig. 2), mAb 5A2 was used for the mapping with the fd phage (landscape) library. The screened phage pool obtained after each round of phage screening were used directly to amplify the recombinant pVIII gene of possibly all the mAb 5A2 binding phage clones by PCR. The PCR amplification was performed with a high-fidelity Taq polymerase in triplicates to account for any amplification bias. Amplicons of about 140bp were obtained from each phage pool triplicate from each biopan Fig. 3. Since the insert size varies between 18 to 36 base pairs within each phage pool the bands observed represents the average size of all the amplicons in a phage pool. Hence a band of 140 bp is consistent in all the samples.

Over 97% of all the reads obtained from the NGS were successfully merged (Table. 1). The total reads obtained for the phage pools ranged between 40,808 and 91,457 as summarized in table 1. These results show that out of 1000 million possible recombinant pVIII fusion peptides sequence

combinations, present in a phage pool, about 50,000 sequences, on average, were read. More than 97% of which, when read in both forward and reverse direction (paired end reads), merged into a single sequence as the overlapped region was identical.

The sequence reads were grouped based on their consensus/ frequencies (f). The sequences that are unique ($f=1$) or recur 2-9 times ($f=2$ to 9), recur between 10-49 times ($f=10$ to 49), 50-99 times ($f=50$ to 99), 100-199 times ($f=100$ to 199), 200-299 times ($f=200$ to 299), 300-350 times ($f=300$ to 350) were grouped as "Consensus 1-9", "Consensus 10-49", "Consensus 50-99", "Consensus 100-199", "Consensus 200-299", "Consensus 300-350", respectively. Any sequences with $f>350$ were not grouped and were analyzed individually. Fifteen such sequences, that were the most abundant phage sequences in the biopan 3 phage pool, were identified and their abundances were traced in each of the preceding biopans i.e. biopans 1 and 2. The average of the sequence abundances/frequencies in triplicates for all of the 15 peptide sequences in each biopan are shown in Fig. 4b and Table 2. The abundances of the each of the selected 15 peptide sequences were less than 10 in the first biopan but increased significantly over the next two biopans except for 5A2-P4, 5A2-P9, 5A2-P11, 5A2-P12, 5A2-P14. These results show that there was a progressive enrichment of most of the mAb 5A2 binding sequences in the consecutive rounds of positive selection.

The percent phage sequence distribution in a biopan phage population was determined by calculating the percent frequency for each of the sequence groups and the 15 selected individual sequences (Figure 4a and c, Table 2). The biopan 1 phage pool mostly (89%) consisted of phages with "Consensus 1-9". The share of this group reduced dramatically to 48% in the second biopan and was ultimately reduced to 14% by the third biopan. Whereas the share of all the individual sequences increased over the consecutive biopans. For example, the sequence 5A2-P1

(CHWEWGSYGIVRTC) was 0% of the biopan 1 phage pool but by the second and third biopan it increased to 0.8% and 4.5%, respectively (Fig. 4c, Table 2). These results highlight the progressive increase in the diversity of the mAb 5A2 binding sequences over the consecutive biopans.

The peptide 5A2-P1 (CHWEWGSYGIVRTC) with the highest % total reads (4.5%) and frequency (2178), and 5A2-P14 (PVHVSAPSAL) with the lowest % total reads (0.73) and frequency (350.6) most likely represent the peptides with most and the least affinities amongst the 15 peptide sequences in biopan 3 (Figure 4b and c, Table 2). The specificities of these peptide sequences for the mAb 5A2 will be tested in a peptide ELISA to identify the sequence that is most likely to represent the mimotope/epitope.

2.3 Validate that peptides corresponding to the phage displayed peptide sequences are specific to BV neutralizing antibodies in a peptide ELISA.

The most abundant mAb 5A2 binding and 4E11 binding recombinant pVIII fused peptide sequences were identified using NGS and phage display respectively. To test which one of these sequences truly represented the epitope/ mimotope of the mAb 5A2 and or 4E11 peptides corresponding to these sequences were synthesized and tested for binding to the mAb 5A2 and compared with binding to a non-specific anti-BV gB mAb 9F1 on a CovaLink plate. To mimic the cyclic peptides expressed on the phages the two cystines present on each cyclic phage sequence were linked with a disulphide bond during the commercial synthesis of the peptides. The peptide specificity was measured as the OD (absorbance at 405-490 nm) produced by the binding of the mAbs. A peptide was considered specific if the difference in the OD_{5A2} and OD_{9F1}, at two or more dilutions of the peptide, was greater than 0.2. The peptides 5A2-P2, P3, P4, P5, P7, P12, P15 and P.4E11.PhD had OD_{5A2} - OD_{9F1} > 0.2 at the peptide dilutions 10 and 5

$\mu\text{g/mL}$, 20, 5 and 1.25 $\mu\text{g/mL}$, 20 and 1.25 $\mu\text{g/mL}$, 5 and 1.25 $\mu\text{g/mL}$, 20, 10, 1.25 $\mu\text{g/mL}$, 5, 2.5, 1.25 $\mu\text{g/mL}$, 2.5 and 1.25 $\mu\text{g/mL}$ and 20-1.25 $\mu\text{g/mL}$ respectively (Table 3).

Since the peptides 5A2-P2, P3, P4, P5, P7, P12, and P15 show $\text{OD}_{5\text{A}2} - \text{OD}_{9\text{F}1} > 0.2$ only at two or three dilutions they probably represent only part of the complete epitope/ mimotope. Conversely, P.4E11.PhD shows $\text{OD}_{5\text{A}2} - \text{OD}_{9\text{F}1} > 0.2$ at all dilutions hence it should be more specific than the other peptides and should be a better representative of the mAb 4E11 and/or mAb 5A2 epitope.

The mAb 5A2 instead of 4E11 was used to measure the peptide activity of the P.4E11.PhD since mAb 5A2 bound much better than 4E11 to phage displayed peptide (of the same sequence) in the pELISA (Figure 2). P.5A2.PhD (Figure 5N) is a peptide of aa sequence CWAPFWSDCGGS identified previously using commercial phage display library. The mAb 9F1 specific control peptide OP2, as expected, was highly specific for the mAb 9F1 and had a minimum cross-reactivity with the mAb 9F1. Collectively, these results show that out of all the peptides that were considered specific for the mAb 5A2 P.4E11.PhD shows the highest specificity and thus should better mimic the mAb 5A2 and/or 4E11 epitope.

2.4 Determine putative location of the epitope/mimotope on BV gB by mapping the epitope to the predicted three-dimensional model of gB.

The peptide sequences tested in the peptide ELISA were tested for the presence of target unrelated motifs using the TUPScan tool. The QSYPL motif which is a confirmed binder to unrelated antibodies was present in the 5A2-P3, 5A2-P9 and 5A2-P11 peptide sequences. Another motif W-x(2)-W which is a confirmed plastic binder was present in the 5A2-P7 peptide sequence. These sequences were not used for further analysis.

A 3D model of BV gB that was prepared by projecting BV gB sequence (uniprot Q7T5E1) on the known crystal structure of HSV1 gB (PDB code 6BM8) as shown in Figure 6 was used to visualize the epitopes [89]. The five domains (Domain I-V) corresponding to those delineated by Heldwein et al were identified and marked on the BV gB model [44]. The BV gB aa residues that correspond to the 5A2-P2, 5A2-P4, 5A2-P5, 5A2-P12, 5A2-P15, P.4E11.PhD sequences were identified using the Episearch webtool [90]. The BV gB model was used as a query model and the 5A2-P2, 5A2-P4, 5A2-P5, 5A2-P12, 5A2-P15, P.4E11.PhD sequences were used as query sequence. The predicted result with the highest possible score of 1 was selected for each query. The query sequence with 5A2-P4 returned two results with score 1 (5A2- P4.1 and 5A2-P4.2). The summary of the Episearch results is shown in the Table 4. The epitope sequence corresponding to peptide 5A2-P2 was mapped to the predicted domain II (Fig 7a), 5A2-P4.1, 5A2-P4.2, P.4E11.PhD, 5A2-P12 to domain III (Fig. 7b,c,e,f) and 5A2-P5 to domains IV and III (Fig. 7d). The peptide 5A2-P15 was mapped to the disordered signal sequence region of the model hence it was not shown in Figure 7. Out of the six predicted epitope sequences mapped on the 3D model five of them map to the domain III and out of the five, four map to same two discontinuous regions of the domain III composed of aa residues 103-120 and aa residues 513-558.

Collectively these results show that the mAbs 5A2 and 4E11 have a common discontinuous epitope that is located on the two discontinuous regions of the predicted domain III of BV gB. Hence, we disproved our hypothesis that stated the mAb 5A2 and 4E11 epitopes are located on fusion loops located in the domain I of gB.

2.5 Aim I Conclusions

The goal of this aim was to test if the neutralizing mAb targeted the fusion loops that are generally found at the base of the herpes virus gB. This base is made up of two domains- domain I and V, as defined by Heldwein et al (Fig.7) [44]. The domain I forms majority of the base and the fusion loops are located on this domain. To test our hypothesis, we identified the mimotopes that represent the corresponding epitope of gB that is targeted by the neutralizing mAbs 5A2 and 4E11 using two distinct phage display libraries and different sequencing approaches to analyze the selected phages from each library. A commercial pIII fused random peptide phage display library and a fd phage (landscape) pVIII fused random peptide phage display library was used to map the epitopes of mAb 4E11 and 5A2 respectively, and sanger's sequencing and NGS, respectively, were used to sequence the selected phages.

The phage display mapping of the gB with the mAb 4E11 yielded only one positive clone despite the three rounds of positive selection which are sufficient to enrich the target (antibody) specific phage population [73, 91]. This could be due to a harsh panning strategy, such as the use of a closely related mAb for negative selection step. The best specificity binders in the original library might have been weeded out by the closely related mAb in the negative selection step leaving only the poor affinity binders. These poor binders may have low specificity for the mAb and may eventually be outnumbered by the high affinity low specificity binders leaving very few of the high affinity high specificity binding clones. The one positive phage clone-20 was more specific for the closely related mAb 5A2 than 4E11 that was used to map the clone, this could be because the 5A2 was developed against a more immunogenic part of the epitope and thus has a higher specificity for the postulated common/proximal epitope of the two mAbs. Besides that,

5A2 may have some conformational advantage over 4E11 that improves its binding with the peptide expressed on the clone.

The use of fd phage (landscape) library when used in conjunction with NGS is a powerful tool as there is about 30-fold more expression of peptide per phage thus providing a lot more opportunities for target binding compared to the commercial pIII fusion phage libraries. The NGS is a highly robust technique that makes it possible to analyze thousands of phage sequences at once. As a result, we were able to analyze about 50,000 sequences mAb 5A2 binding phage sequences on average per biopan per replicate.

Since the peptide function (i.e. recognition of the target antibody) is directly dependent on the accuracy of its DNA sequence, we had to ensure that there was minimum error in the amplification of the fusion peptide DNA sequences and NGS. To this end we used a high fidelity DNA polymerase, to avoid incorporation of mismatched base pairs, we kept the PCR cycles to a minimum (15 cycles total) to further reduce the chances of any error made by the polymerase and we performed PCR amplification for each phage pool in triplicates and then each of these triplicates was sequenced individually. As evident from Fig. 5 and Table 2 there was not a single mismatch in the 15 sequences that were selected based on their abundance. Also, the small size of the error bars in Fig. 5 b there shows that there was a minimum difference in the abundance of the each of the 15 sequences within the triplicates in each biopan.

The Fig. 5b compares the average of crude frequency values of each of the 15 selected sequences within the three biopans whereas Fig. 5c compares the average of frequencies of each sequence in relation to the total reads of that sample (% of total reads) within the three biopans. Hence it is a more accurate depiction of the differences in the phage sequence frequencies between biopans. It is also worth noticing that the pattern of the graph showing the crude

(average) sequence frequencies (Fig. 5b) is almost identical to the graph showing the percent of total reads (Fig. 5c) even though the total reads for each replicate of each biopan are different (Table 1). This is only possible if the sequence: total reads ratio remains constant throughout the replicates. Hence this shows that our sequencing results were highly accurate.

Since, the binding of the 5A2 specific peptides to mAb 5A2 was compared with that of a high affinity anti-BV gB mAb 9F1, this was a stringent comparison. Hence, wherever the difference between the peptide binding with mAbs 5A2 and 9F1 ($OD_{5A2} - OD_{9F1}$) was greater than 0.2 for at least two dilutions of the peptides, the peptides were be considered specific to the mAb 5A2.

The magnitude of the difference observed in the activity of the mAbs for their respective peptides was observed to be quite significant. The mAb 9F1 had much higher binding with its peptide OP2 ($OD \sim 4$), whereas mAb 5A2 binding with its corresponding peptides (e.g. P.4E11.PhD) was significantly less ($OD \sim 0.8$). If the quality and similarity of the peptide with the mAb epitope of the synthesized peptides are anticipated to be similar, as both peptide sequences were identified using the same methods, then the low binding of mAb 5A2 and the high binding of mAb 9F1 could be due to their respective low and high affinities.

The synthesized peptides represent the actual fusion peptides that were expressed on the phages. The binding of the peptides 5A2-P2, 5A2-P4, 5A2-P5, 5A2-P12, P.4E11.PhD to the mAb 5A2 was higher than their binding with a high affinity BV gB mAb 9F1. This confirms that these peptides were specific for the mAb 5A2. When mapped to the 3D model of the BV gB the sequences of peptide 5A2-P2 and peptides 5A2-P4, 5A2-P5, 5A2-P12, P.4E11.PhD correspond to the domain II (the middle region) and domain III (the core region) respectively. The domain III contains three discontinuous segments aa residues 104-120, 487-559 and 648-656. Most of

the domain III representing peptides are concentrated in the short segments of 113-120 and 516-526, and only one of them (5A2-NGS5) corresponds to 528-609.

Hence, we can conclude that the neutralizing mAbs 5A2 and 4E11 are at least partially specific for a discontinuous epitope, aa residues 113-120 and 516-526, located at the domain II of BV gB.

2.6 Tables

Table 1. Summary of Next Generation Sequencing of the screened phage pools

Sample ID	Merged Reads	Not merged reads	Total Reads	Merged Over Total (%)
5A2-B1A	45,768	1,057	46,825	97.74
5A2-B1B	47,466	1,145	48,611	97.64
5A2-B1C	46,520	1,174	47,694	97.54
5A2-NSA	50,770	1,210	51,980	97.67
5A2-NSB	78,884	1,920	80,804	97.62
5A2-NSC	88,784	2,672	91,457	97.08
5A2-B2A	42,013	1,238	43,251	97.14
5A2-B2B	41,285	1,034	42,319	97.56
5A2-B2C	39,140	813	39,953	97.97
5A2-B3A	46,141	1,085	47,226	97.70
5A2-B3B	44,998	1,060	46,058	97.70
5A2-B3C	47,129	1,095	48,224	97.73
5A2, Monoclonal antibody 5A2; B1, Biopan 1; B2, Biopan 2; B3, Biopan 3; NS, Negative selection; A/B/C, triplicates A,B and C				

Table 2. The frequencies of selected mAb 5A2 binding peptide sequences

Peptide ID	aa Sequences	Biopan 1		Biopan 2		Biopan 3	
		<i>f</i>	% of total reads	<i>f</i>	% of total reads	<i>f</i>	% of total reads
Consensus 1-9	---	42,232.51	89.10	8671.50	48.10	6977.33	14.55
Consensus 10-49	---	2,038.16	4.30	8003.33	18.41	9070.67	18.91
Consensus 50-99	---	161.16	0.34	4720.50	10.79	4418.00	9.33
Consensus 100-199	---	0.00	0.00	5454.00	12.47	5798.67	12.09
Consensus 200-299	---	0.00	0.00	2715.33	5.46	3394.33	3.08
Consensus 300-350	---	0.00	0.00	1461.00	3.73	6977.33	3.98
5A2-P1	CHWEWGSYGIVRTC	1.33	0.00	355.33	0.80	2178.33	4.54
5A2-P2	IRTASTQYRPPS	0.67	0.00	467.33	1.09	1893.33	3.95
5A2-P3	CTQSYSPDC	0.33	0.00	554.33	1.28	1234.33	2.57
5A2-P4	CLPYFTIPQC	0.00	0.00	1691.33	4.06	1173.00	2.45
5A2-P5	CPGRAAGVFLSELC	0.33	0.00	284.67	0.68	1127.00	2.35
5A2-P6	VRHVSITSTH	0.00	0.00	113.67	0.27	1130.00	2.36
5A2-P7	CEYREWFPWL VHSC	0.33	0.00	817.00	1.88	1170.00	2.44
5A2-P8	TWWQYSFAEKHP	0.33	0.00	200.00	0.46	939.33	1.96
5A2-P9	TQSYPPPS	1.00	0.00	2114.33	4.94	875.33	1.82
5A2-P10	ASLPGNPPYPPL	1.00	0.00	429.67	0.99	822.33	1.72
5A2-P11	PVRTQSYPNPEL	0.67	0.00	986.00	1.29	752.00	1.57
5A2-P12	CPPRWCETLP	2.00	0.00	990.67	2.39	528.67	1.10
5A2-P13	CPGFDVGLIPSC	0.00	0.00	126.67	0.28	383.67	0.80
5A2-P14	CGTFTPC	5.33	0.01	495.33	1.18	350.67	0.73
5A2-P15	PVHVSAPSAL	0.67	0.00	100.50	1.34	356.33	0.74

5A2, Monoclonal antibody 5A2; P1- P15, Peptide 1-15; AA, Amino acid; *f*, frequency; Sequence frequencies and the percentage of total reads shown are the mean values of sequence frequencies and percentage of total reads obtained from the sequencing of triplicates of phage amplicons from each biopan.

Table 3. Difference in OD_{5A2} – OD_{9F1} in a direct binding Covalink peptide ELISA

Peptide ID	OD _{5A2} – OD _{9F1}				
	20	10	5	2.5	1.25
5A2-P2	0.139	<u>0.294</u>	<u>0.256</u>	-0.052	-0.219
5A2-P3	<u>0.201</u>	0.05	<u>0.288</u>	0.015	<u>0.392</u>
5A2-P4	0.2	0.149	0.11	0.193	<u>0.529</u>
5A2-P5	0.115	0.029	<u>0.228</u>	0.131	<u>0.249</u>
5A2-P6	-0.109	-0.079	-0.244	0.05	-0.04
5A2-P7	<u>0.281</u>	<u>0.241</u>	-0.035	0.089	<u>0.293</u>
5A2-P8	-0.163	-0.138	0.02	-0.09	-0.129
5A2-P9	-0.007	0.037	0.014	0.177	<u>0.379</u>
5A2-P10	0.013	0.061	<u>0.328</u>	0.075	0.153
5A2-P11	<u>0.282</u>	-0.171	0.048	-0.023	0.146
5A2-P12	0.03	0.03	<u>0.305</u>	0.203	<u>0.476</u>
5A2-P14	-0.115	0.035	<u>0.274</u>	0.008	0.053
5A2-P15	-0.091	-0.131	-0.013	0.346	<u>0.24</u>
5A2 PhD	-0.165	0.043	0.101	0.041	0.124
4E11 PhD	<u>0.226</u>	<u>0.358</u>	<u>0.347</u>	<u>0.306</u>	<u>0.362</u>

OD_{5A2}, absorbance by 5A2 binding; OD_{9F1}, absorbance by 5A2 binding ; 5A2-P2-P15, mAb 5A2 selected peptides 2-15; P.5A2.PhD, mAb 5A2 selected peptides using phage display; P.4E11.PhD, mAb 4E11 selected peptide using phage display; The OD_{5A2} - OD_{9F1} > 0.2 are underlined. The peptides having OD_{5A2} - OD_{9F1} > 0.2 at more than two dilutions of the peptide are shown in bold.

Table 4. Episearch results summary: Mapping of conformational epitopes

Peptide ID	aa Sequences	Predicted Residues	Score	Predicted Length
5A2-P2	IRTASTQYRPPS	R386 , T387 , Y389 , A390 , S392 , R393 , Q425 , Y428 , Q459 , R461 , R462 , P463 , A466 , A467 , T469 , P470 , P472 , S473 , A474	1	19
5A2-P4.1	CLPYFTIPQC	F114 , Q116 , P117 , P119 , C120 , I513 , C516 , L518 , Q519 , L523 , T524 , L525	1	12
5A2-P4.2		Q113 , F114 , Q116 , P117 , C516 , L518 , Q519 , L523 , T524 , L525 , I537 , T541	1	12
5A2-P5	CPGRAAGVFLSELC	C103 , P105 , P106 , V111 , V112 , E528 , A529 , R530 , L532 , P534 , A536 , S539 , A540 , V542 , R549 , L551 , G552 , V554 , V557 , S558 , E606 , E609	1	22
5A2-P12	CPPRWCETLP	E115 , P117 , R118 , P119 , C120 , W515 , C516 , E517 , L518 , E522 , L523 , T524 , L525 , W526	1	14
5A2-P15	PVHVSAPSAL	P7 , L8 , P9 , L10 , P11 , S12 , P13 , L14 , V15 , P16 , L17 , L18 , A19 , A21 , V622	1	15
P.4E11.PhD	CEPFWTNCGGGS	F114 , E115 , P117 , P119 , C120 , W515 , C516 , E517 , N520 , E522 , T524 , W526 , N527	1	13
5A2-P2-P15, mAb 5A2 selected peptides 2-15; P.5A2.PhD, mAb 5A2 selected peptides using phage display; P.4E11.PhD, mAb 4E11 selected peptides using phage display; aa, amino acids				

2.7 Figures

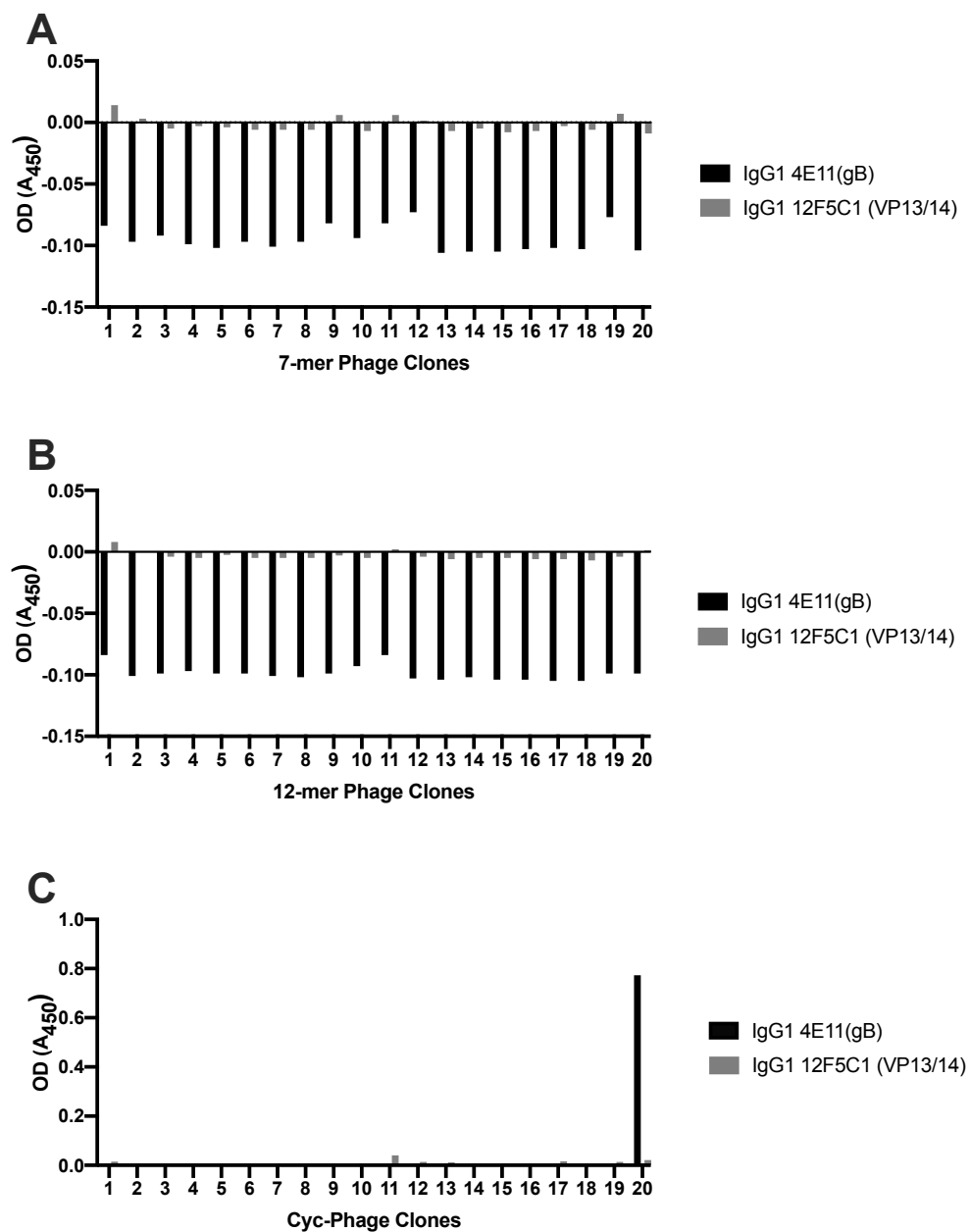


Figure 1. Immunoreactivity of selected phage clones expressing random peptides with mAb IgG1 5A2 in a phage enzyme-linked immunosorbent assay (pELISA).

Twenty phage clones with **(a)** 7-mer, **(b)** 12-mer, and **(c)** random cyclic peptide displays were randomly selected after three rounds of biopanning and detected using anti-BV gB mAb 5A2 and anti-BV VP13/14 mAb 12F5C1 (negative control) on a in a pELISA .

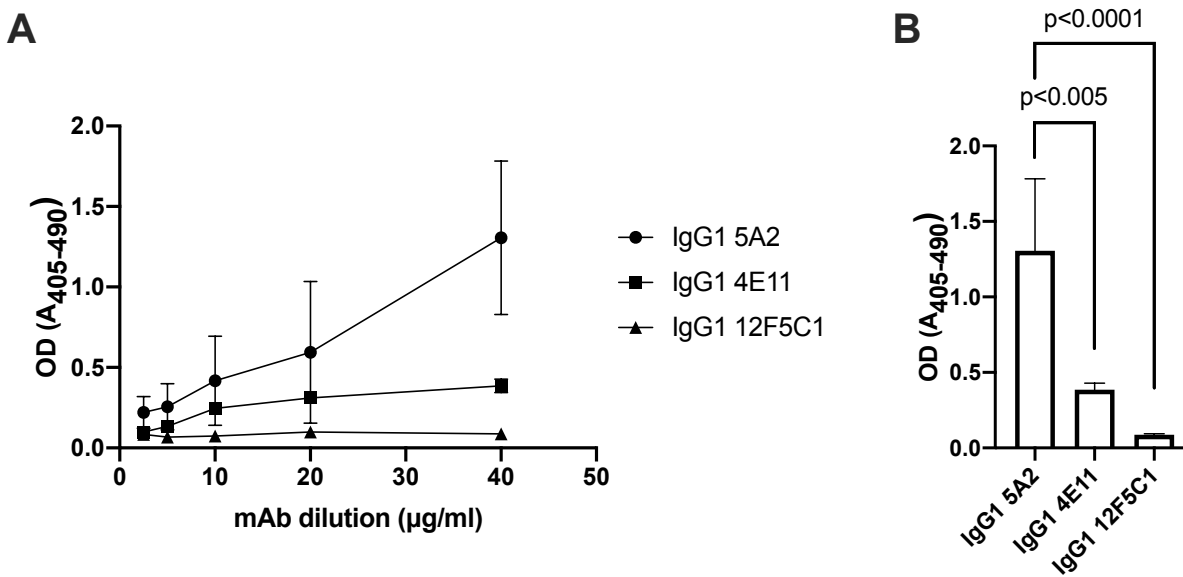


Figure 2. Testing the capture of mAbs by phage clone coated on an ELISA plate.

A phage clone selected from pELISA was tested for its ability to capture B virus neutralizing mAbs. Panel (a) shows the capture of two-fold diluted (40μg/mL- 2.5μg/mL) B virus neutralizing mAbs IgG1 5A2 and 4E11 and a control mAb 12F5.C1 by 1.00E+11 PFU/mL phage clone 20 selected by mAb 4E11. Panel (b) compares the amount of mAbs captured at the dilution of 40μg mAb/mL by the 4E11 phage clone. The OD values represent average mean values from duplicate tests.

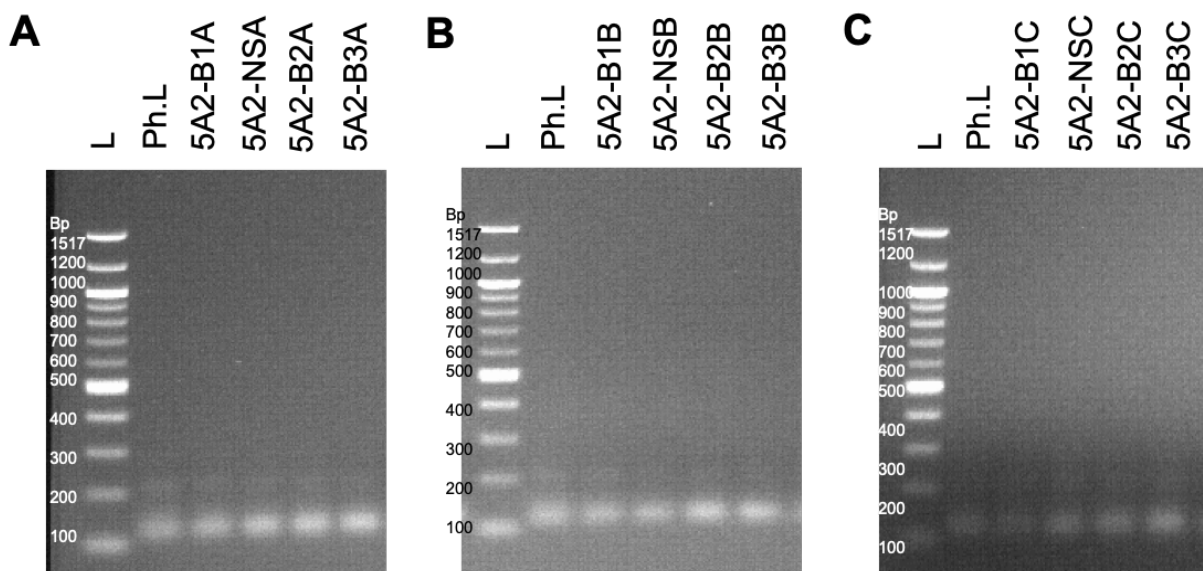


Figure 3. Validation of the size of the recombinant pVIII fusion peptide expressing amplicons.

The recombinant pVIII fusion peptide expressing gene from the phage pools obtained from the four rounds of biopanning and an unscreened library were amplified by PCR in triplicates (**a**, **b**, **c**). The approximate size of the amplicons from each PCR replicate was determined by gel electrophoresis.

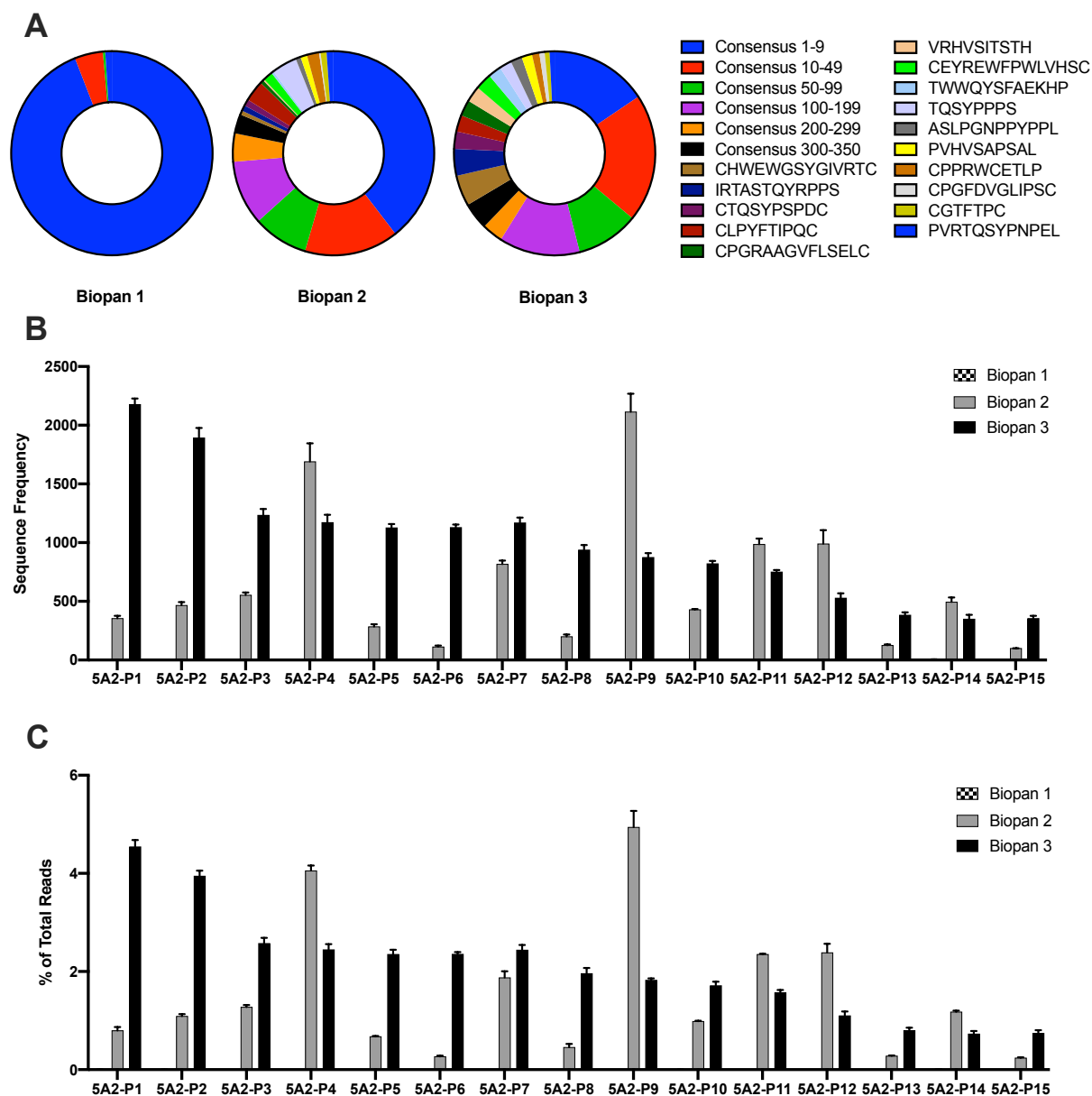
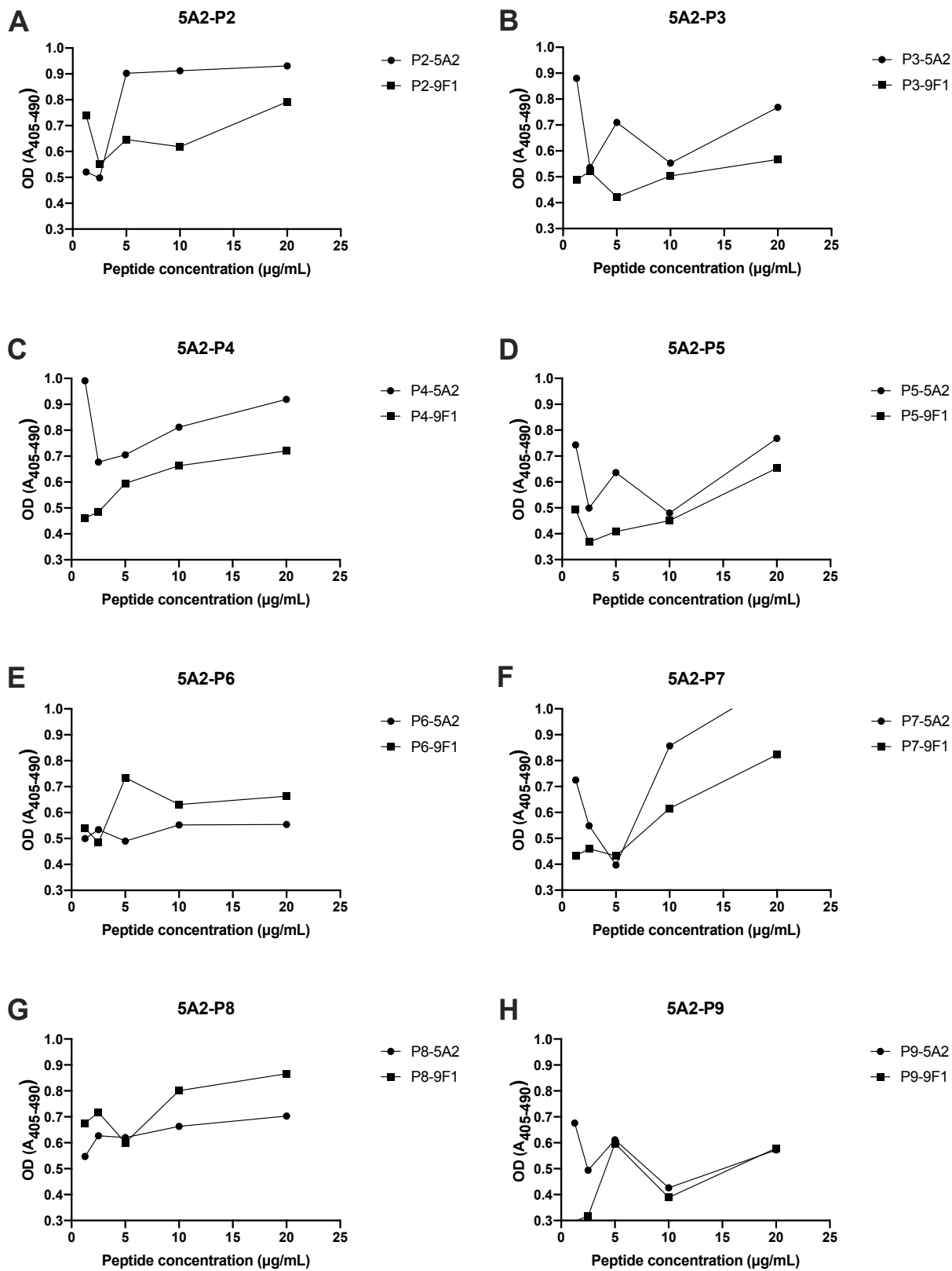


Figure 4. Enrichment of IgG1 5A2 binding aa sequences in consecutive rounds of biopanning.

After three rounds of positive selection biopanning against mAb 5A2 the phage pools eluted from each round show progressively increased selection of mAb 5A2 binding phage sequences. The sequences that are unique, recur 2-9 times (consensus 1-9), sequences that recur between 10-49 times (Consensus 10-49), sequences that recur between 50-99 times (Consensus 50-99), sequences that recur between 101-195 times (Consensus 101-195), sequences that recur between

206-284 times (Consensus 206-284), sequences that recur between 303-431 times (Consensus 303-431), and fifteen sequences with the highest number consensus amongst all the reads are shown. Panel **(a)** shows the percent (of total reads) distribution of mAb binding sequences for each biopan. Panel **(b)** compares the average of crude frequency values of each of the 15 selected sequences within the three biopans whereas **(c)** compares the average of frequencies of each sequence in relation to the total reads of that sample (% of total reads) within the three biopans.



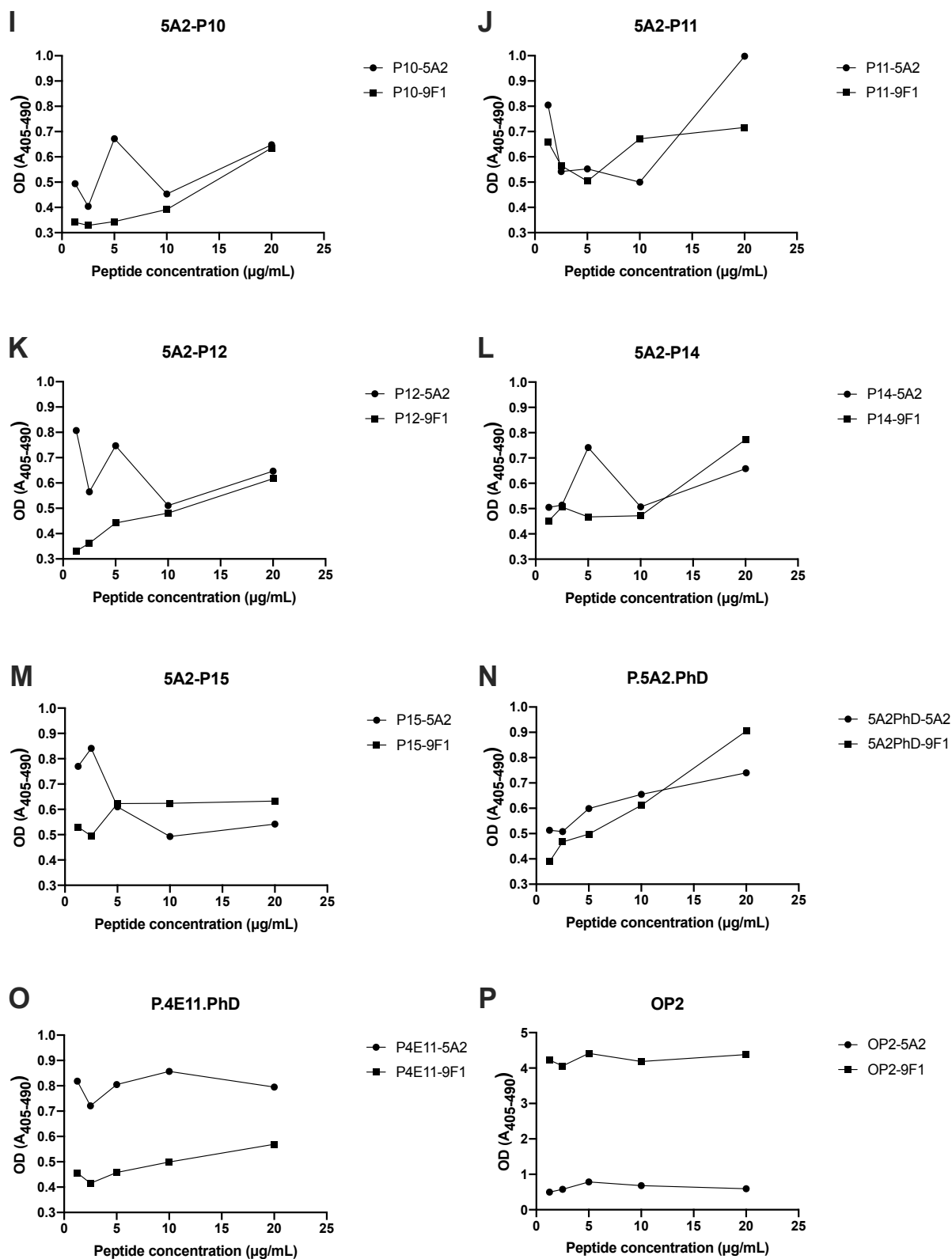


Figure 5. Measuring the peptide activity in a direct binding assay.

To validate that the peptides (5A2 P2-P12 and 5A2 P14,P15, P.5A2.PhD, P.4E11.PhD) identified by the phage display epitope mapping represent/mimic the epitope(s) of the mAb 5A2 and 4E11 each of the synthesized peptides (**a-p**) diluted two-fold dilution from 20-1.25 $\mu\text{g/mL}$ were tested for their binding to the mAb 5A2 and 9F1 by using the peptides as an antigen in a direct ELISA. The binding of peptides with mAb 5A2 and 9F1 was compared for each peptide. (**p**) A peptide OP2 with known specificity for mAb 9F1 was tested for its binding with both mAbs 5A2 and 9F1.

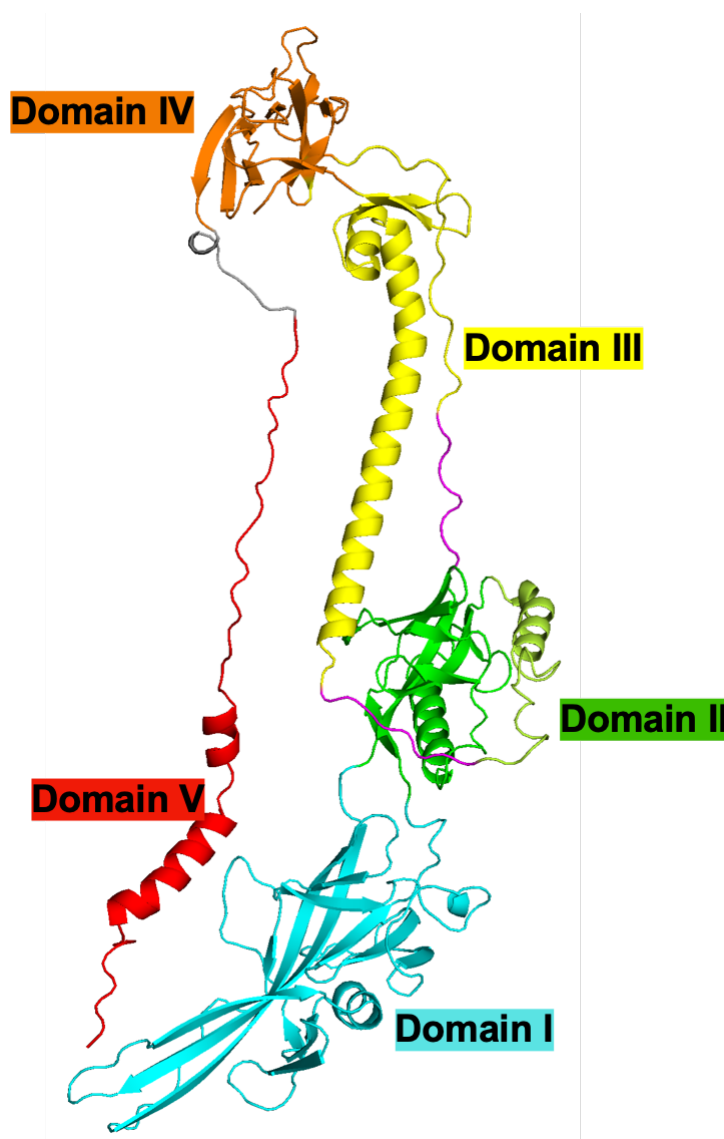


Figure 6. A three-dimensional structure of a full-length BV gB monomer.

A 3D model of the monomer of gB with Q (stability value)= -4.84 is shown. Domain I (residues 141-350) is shown in blue, linker regions (residues 121-128; 479-486) in magenta, Domain II (residues 129-140; 351-446) in green, domain II- disordered region (residues 447-478) in light green, domain III (residues 104-120; 487-559; 648-656) in yellow, domain IV (residues 98-103; 560-647; 560-647) in orange, domain V (residues 657-712) in red and the membrane proximal region (MPR), transmembrane domain (TMD) and cytoplasmic domain (CTD) (residues 713-891) are not shown for simplicity.

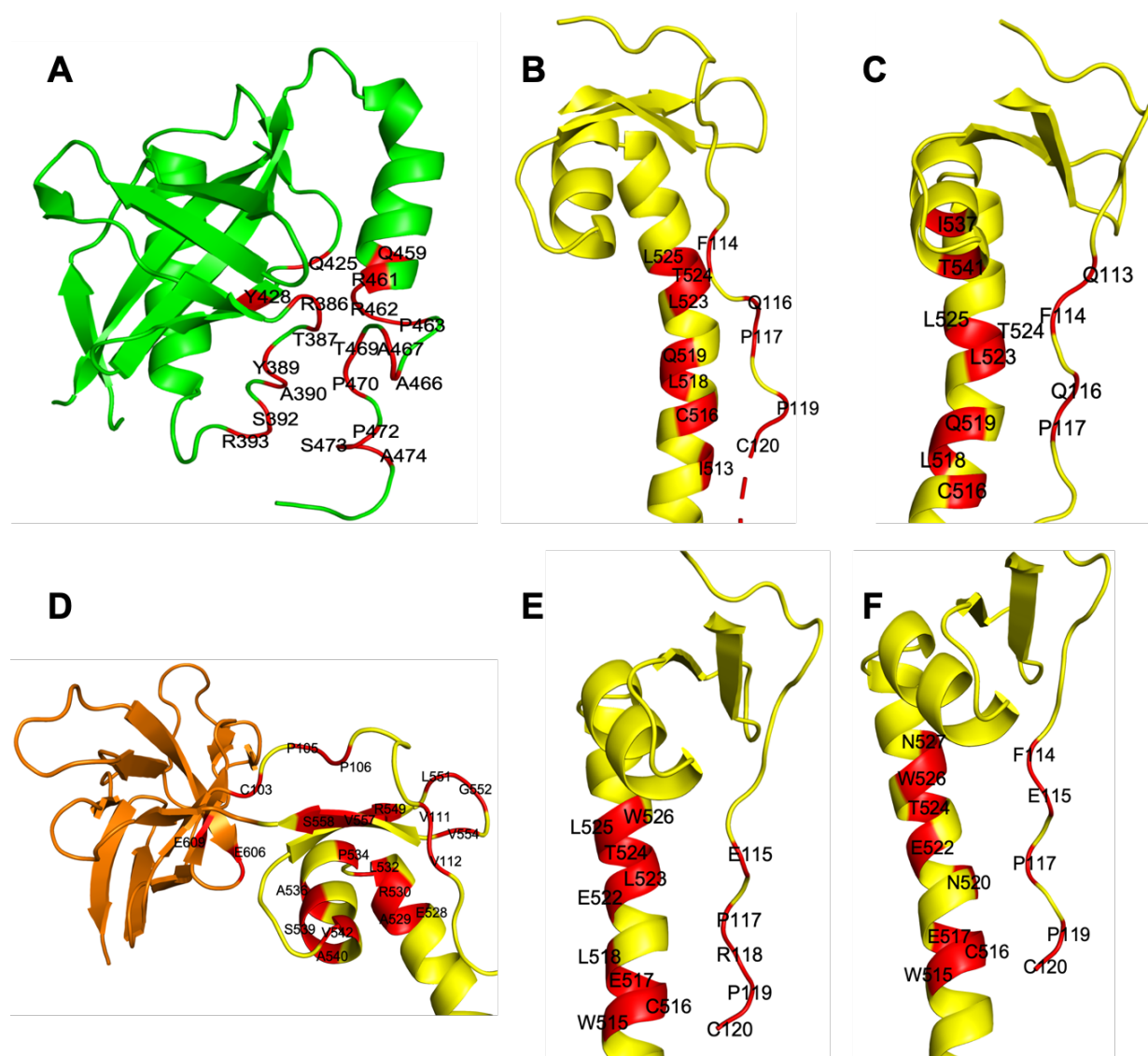


Figure 7. Mapping of conformational epitopes to the predicted three-dimensional model of BV gB.

The location of mimotope sequences corresponding to the peptides, that showed high binding with the mAb 5A2, on BV gB is shown. The peptides 5A2-P2 (IRTASTQYRPPS) was mapped to the predicted domain II **(a)**, 5A2-P4 (CLPYFTIPQC) panel **(b)** and **(c)**, P.4E11.PhD (CEPFWTNCGGGS) panel **(e)** and 5A2-P12 (CPPRW CETLP) to domain III **(f)** and P5(CPGRAAGVFLSELC) to domain IV and III **(d)**.

3 AIM II: TEST IF SPECIFIC gB EPIOTOPE(S) INDUCE UNIQUE, B VIRUS-SPECIFIC HUMORAL RESPONSE(S) DIFFERENTIABLE FROM THOSE INDUCED BY CLOSELY RELATED MEMBERS OF THE VIRUS SUBFAMILY.

B virus (BV, *Macacine alphaherpesvirus 1*) is enzootic in macaque monkeys, but zoonotic infection in humans cross reacts with other alpha herpesviruses [60, 92, 93]. The cross reactivity is due to the shared homologous genes between B virus and human herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) [2]. There is about 62% similarity in aa acid sequences between all homologous proteins of B virus and both HSV-1 and HSV-2[65]. The currently available diagnostics that rely on detecting the levels of these antibodies induced by BV fail to differentiate whether antibodies in humans are specific for BV in the presence of high levels of anti-HSV antibodies[42, 80, 94]. In order to overcome this challenge, it is critical to identify the unique epitopic markers of the B virus to be able to induce specific humoral immune responses. A random peptide phage display epitope mapping and custom BV gB peptide array epitope mapping will help us identify novel BV specific epitopes that can be used to increase the sensitivity and specificity of a BV serological test for humans. The phage screening can help us identify linear, conformational or discontinuous epitopes, whereas the overlapping peptide array is a high throughput assay and can identify linear epitopes.

3.1 Identify epitope sequences recognized by BV specific mAb by random peptide phage display library.

To probe for continuous and/or discontinuous epitopes of the mAb 9F1 we screened the phage displayed 7-mer, 12-mer and a loop-constrained heptapeptide cyclic random peptide libraries. After three rounds of biopanning, carefully selected, phage clones were randomly picked from 7-mer, 12-mer, and cyclic third round eluate titration plates prior to amplification.

Subsequently, each clone was amplified and evaluated for its activity with the mAb 9F1 and a non-specific mAb G12D122 (negative control) in phage ELISA (Fig. 8). We observed that all twenty 7-mer clones (1-20), eighteen of the twenty 12-mer phage clones (clones 10 and 20 did not react) and thirteen of the twenty cyclic phage clones were recognized by mAb 9F1. None of the selected phage clones reacted with the mAb G12D12. Sequencing of the positive phage clones revealed consensus sequences of 1)“SWRPGDYGGG”, 2)“ANWSHWEFNRRPGGG”, and 3)“ACWRPGDMACGGG” within the 7-mer, 12-mer, and Cyc phage clones, respectively (Table 5). The high redundancy of 41%, 69% and 94% observed in the sequences of the randomly selected 7-mer, 12-mer, and cyclic- clones signifies a high degree of sufficient enrichment by the third and final round of selection, eliminating the need of a fourth selection round. The consensus motif “RPGD” of the 7-mer and Cyc- phage clones and the consensus motif “RRPG” of the 12-mer phage clones corresponded to BV gB sequence of amino acids (aa) 461-465. The 7-mer, 12-mer, and Cyc- phage clones had an overall consensus motif “RPG”. The 7-mer phage clones 10, 11, and 12, and the 12-mer phage clone 16 had OD >0.6 (A_{450}), but could not be sequenced due to low DNA concentrations. Peptides biotinylated at the carboxyl terminal corresponding to the 7-mer, 12-mer, and cyclic phage clone consensus sequences were subsequently synthesized. Phage display sequencing results provided an idea of the approximate size (10-15 aa) and motif (RRPGD) to search for within the BV gB sequence. Hence, we synthesized another biotinylated peptide, OP2 Mod, BVgB sequence aa 457-468, “REQERRPGDAAA”. This peptide matched the characteristics of mAb 9F1-recognized epitope as defined by the phage display as it contains the consensus motif “RRPGD” and was 12 aa in length.

3.2 Identify epitope sequences recognized by BV specific mAb by peptide array.

To identify the complete linear epitope which binds with mAb 9F1, the BV gB overlapping peptide array was employed. The peptides were spotted on a chip with four subgrids, on each subgrid either 90nmol, 180nmol, 270nmol or 460nmol of peptide per spot were spotted in duplicates (Fig. 10a). The four different concentrations of peptides were optimized to minimize signal-to-noise-ratios for the positive signals. Results revealed that mAb 9F1 bound to only a single 24-aa region of BV gB sequence, aa 451-474, represented by the four overlapping peptides with a consensus sequence ERRPGD (Fig. 10d). The fluorescence intensity corresponded to the amount of mAb 9F1 reacting with the peptides. As predicted, the fluorescence, and thus mAb binding, increased with increasing concentrations of the overlapping peptides, suggesting that the peptide spot does not saturate with peptides even until 360 nmol (Fig. 10b). OP1 demonstrated the highest activity as it had the highest mean fluorescence amongst the four overlapping peptides. There was a significant difference in the mean fluorescence intensities of the OP1 with OP2, OP3 and OP4 ($p < 0.05$) at the peptide concentration of 270 nmol/spot (Fig. 3C). At other concentrations of the peptides (360, 180 and 90 nmol) there was no significant difference ($p > 0.05$) between the four overlapping peptides. These results suggest that each of one of these peptides represents the epitope of the mAb 9F1, OP1 having a slightly better activity than the rest of the peptides.

3.3 Validate that peptides corresponding to the unique BV gB motifs identified by 9F1 are specific to 9F1 in a CIBE.

To establish that the peptides truly represent the BV epitope identified by the mAb 9F1, each of the phage display identified peptides (PhdPs 1-3) and OP2 Mod1 peptide were tested for

their ability to block the binding of the B virus specific mAb 9F1 and a control mAb 7G8 to the B virus whole antigen (Fig. 9). PhdP1, PhdP2, PhdP3 and OP2 Mod blocked a maximum of 75.23%, 66.29%, 63.29% and 72.62 of mAb 9F1 binding respectively (Fig. 9c). Whereas, the maximum blocking of the control mAb 7G8 binding to the B virus whole antigen by PhdP1, PhdP2, PhdP3 and OP2 Mod1 was only 34.51%, 32.10%, 19.14% and -14.47% respectively, at 12.5 µg of peptide/mL (Fig. 9d). This shows that all the peptides represent/mimic the target of the mAb 9F1 and not the control mAb 7G8. The maximum blocking of 9F1 binding by OP2 Mod1 could be achieved at a low concentration of just 25µg/mL whereas 100µg/mL of peptide was required to achieve similar levels of blocking by PhdP1-3.

Overall, amongst the PhdPs, PhdP1 exhibited the best sensitivity and PhdP3 exhibited the best specificity for the mAb 9F1. The OP2 Mod1, even at low concentration of 12.5ug/mL, has a comparable sensitivity to PhdP1 and PhdP3 but much better specificity (Fig. 9c, e). This is probably due to the fact that the peptide with aa sequence closest to the BV gB sequence, i.e. OP2 Mod1, is more efficient at blocking compared to PhdP1-3 that only partially resemble the BV gB sequence. Hence these results show that the OP2 Mod1 peptide represents the epitope recognized by mAb 9F1 more accurately than the PhdPs 1-3. The comparatively higher activity of OP2 Mod1 is further evident in the direct binding ELISA (Fig. 11)

3.4 Validate that peptides corresponding to the unique BV gB motifs identified by 9F1 are specific to 9F1 in a direct binding assay

To determine the best configuration of aa residues for optimal epitope function we synthesized C-terminally biotinylated peptides from the available phage clone, peptide array and modified peptide array sequences, and tested their activity in direct ELISA. The activity between

the peptides was compared by measuring the difference in the absorbance at 1:6250 dilution of mAb (Figure 11d-f and Table 6). The overlapping peptides, OP2, OP3 and OP4 exhibited comparable activity (Fig. 11a,d) which was the highest amongst all the peptides tested (Fig. 11a-c, Table 6). Although insignificant ($p>0.05$), OP2 peptide shows marginally better activity than OP3 and OP4. Contrary to the results obtained from peptide activity comparison in figure 11c, OP1 shows the least activity out of the four overlapping peptides. OP2's activity was significantly higher ($p<0.05$ - $p<0.0001$) than all the phage display peptides (PhdP1-3). Amongst the PhdPs, PhdP3 exhibited the highest activity (Fig. 11d).

To test if the N terminal aa of OP1 were responsible for the low activity of the peptide we performed a conservative replacement by substituting six N terminal aa residues-YVRELL of OP1 with aa residues of similar hydropathy indices-FLHDVV. This further reduced OP1 activity, suggesting that the peptide originally existed in the best possible configuration and the modification caused an adverse functional and or structural change (Fig. 11e). The removal of the N terminal motifs "ELL" from the OP2 peptide sequence to get the peptide OP2 Mod1 sequence insignificantly ($p>0.05$) reduced the activity of the OP2 Mod. However, the removal of the last six C terminal aa "AAATPK" from OP2 to get OP2 Mod2 significantly ($p<0.0001$) reduced the activity of OP2 Mod2 highlighting the importance of the C terminal sequences for the optimal epitope function.

The activity of the CS peptide that had only the peptide array consensus sequence "ERRPGD" was significantly lower ($p<0.0001$) than the OP2. By adding three Alanines to the N terminus and six to the C terminus of the CS sequence we created a 15 aa long peptide CS Mod1 which had significantly higher activity ($p<0.0001$) than CS (Fig. 11f, Table 6). Although significantly

different, CS Mod1 had activity similar to the OP2 ($p < 0.05$) suggesting that the motif “AAA” and the size of the peptide are critical for its activity.

We wanted to test if the “ER” aa of the CS peptide are important for the peptide activity hence we replaced the aa with “AA” and restored the size of the peptide to 15aa by adding additional five and seven Alanines to the N terminal and six and three Alanines to the C terminal to get CS Mod2 and CS Mod3. Both these modifications further reduced the activity of the peptide compared to the CS peptide (Fig. 11f). Hence suggesting that “ER” motif, “AAA” motif along with the size of peptide is critical for peptide activity.

3.5 Mapping the epitope ELLREQERRPGDAAA to the three-dimensional structure of gB.

Since no structural information about the B virus glycoprotein B (BVgB) is available, a 3-dimensional model of a monomer of BVgB was prepared by projecting BVgB sequence on the x-ray crystallographic structure of full-length gB from a closely related HSV1 homolog (PDB code 6BM8) [89]. Five different models were generated from the program I-TASSER and were tested for protein structure stability with QMEANDisCo. The stability (Q values) were -4.84, -4.99, -5.29, -5.67, and -7.66 for each of the model 3, 5, 2, 4 and 1. A model, of the highest Q value of -4.84, shown in Figure 5, was selected for the mapping of the most responsive epitope (residue 454-468) ELLREQERRPGDAAA corresponding to the peptide OP2 (Fig. 11) highlighted in green in Fig. 12. The “RPGD” motif, identified by phage display, mapped to residues 461-465 of BV gB (highlighted in red in Fig 12), is integral part of the mapped epitope. It forms a loop that pokes out of the predicted domain II (residues 129-140, 351-446, 447-478; as described for HSV1 gB by Heldwein et al[44]) which might be the most surface accessible area

of the epitope as evident in Fig. 5B. Other high response epitopes corresponding to OP1, OP3 and OP4 peptides (residues 451-465; 457-471 and 460-474, respectively) were all mapped to the predicted domain II in the proximity of OP2 epitope but are not shown in the figure 12 for simplicity. The location of the epitope on the “exposed” surface of BV gB as shown in figure 5B and as depicted earlier on a HSV1 gB model [95] suggests that it is accessible for interaction with B cell receptors and is most likely a B-cell epitope.

3.6 Validating the specificities of the peptides, identified by phage display, for anti-BV and anti- herpesvirus polyclonal antibodies in a direct binding enzyme linked immunosorbent assay.

To test the hypothesis that the peptides identified by phage display epitope mapping are specific to BV antibodies the PhDPs (1-3) and the peptide OP2 Mod1 that was discovered from alignment of PhDPs consensus sequence with BV gB were tested for their binding with macaque and human anti-BV and human anti-HSV antibodies in a peptide coated ELISA. As expected, all the peptides showed high binding ($OD \sim 4$) with the mAb 9F1. Although PhDP1 had significantly lower binding ($p < 0.05$) to 9F1 than the other peptides. OP Mod 1 was significantly ($p < 0.05$) better at capturing anti-BV antibodies from rhesus BV+, cynomolgus BV+ serum pools than the PhdPs and it showed minimal reactivity with rhesus and cynomolgus BV- serum pools (Figure 13a). PhdP1 was significantly better at binding to anti-BV antibodies from all BV infected humans than other peptides ($p < 0.005$). But it also bound to rhesus BV- ($p < 0.05$), cynomolgus BV- ($p < 0.0001$) and Human BV- serum ($p < 0.05$), HSV1+ pool ($p < 0.0001$) and HSV2+ pool ($p < 0.0001$) significantly more than any other peptide (Fig. 13b). Although it was significantly better ($p < 0.005$) than any other PhDP in capturing cynomolgus BV+ Abs it bound to least

amount of Abs from the rhesus BV⁺ pool. PhdP3 showed the most amount of binding with the rhesus BV⁺ sera amongst all the PhdPs.

Collectively, these results show that PhdP1 is sensitive but not specific for BV antibodies in humans. Whereas OP2 Mod1 is both specific and sensitive for BV antibodies in rhesus and cynomolgus macaques, and humans. Although, in humans, its sensitivity is lower than that of PhdP1.

3.7 Validating the specificities of the peptides, identified by peptide array and compound mutagenesis studies, for anti-BV and anti-herpesvirus polyclonal antibodies in a direct binding enzyme linked immunosorbent assay

To test if the peptides OP2, CSMod2, and the HSV1 and HSV2 correlates of OP2, HSV1-OP2 and HSV2-OP2 respectively, are specific for anti-BV and anti-herpesvirus polyclonal antibodies the peptides were tested in a peptide coated direct ELISA. Both OP2 and CS Mod 1 are specific and sensitive for the Rh BV⁺ and cynomolgus BV⁺ serum (Fig. 14a). OP2 overall has better sensitivity and specificity for human BV⁺ sera. OP2 binds significantly ($p < 0.005$) more with Hu BV⁺ (BP) and insignificantly more than the Hu BV⁺ (LS) but CS Mod1 binds marginally better to Abs from Hu BV⁺ (CT) and Hu BV⁺ (SZ). OP2 does not cross react with human either of the BV⁻ sera, HSV1⁺ pool, two of the three individual HSV1⁺ sera, HSV2⁺ pool and two of the three individual HSV2⁺ sera (Fig. 14b,c). CS Mod 1 on the other hand has a significantly more cross-reactivity with one of the human BV⁻ serum ($p < 0.05$), and HSV2⁺ pool and individual sera ($p < 0.05$) compared to OP2. Hence these results suggest that OP2 has better sensitivity and specificity than CS Mod 1 for human BV⁺ sera.

HSV1-OP2 and HSV2-OP2 do not cross-react with any macaque BV⁺ sera but do cross react with two of the five human BV⁺ sera and one of the human BV⁻ sera each of which were both

HSV1 and HSV2 negative. As expected, the HSV1-OP2 reacted with HSV1+ pool and all the HSV1+ individual sera but it did cross-react with two of the individual HSV2+ sera without showing any cross-reactivity for the HSV2+ pool. These results suggest that HSV1-OP2 is specific and sensitive for HSV1+ sera but can cross-react with BV+ sera and show mild cross-reactivity for HSV2+ sera.

HSV2-OP2 peptide cross-reacts with HSV1+ pool and all individual sera and reacts with two out of three individual HSV2+ sera but surprisingly does not react with HSV2+ pool. These results suggest that HSV2-OP2 has poor sensitivity and specificity for HSV2+ sera.

3.8 Determining the sensitivity and the specificity of OP2 peptide for Macaque sera in a direct binding assay

Since in the previous experiments the peptide OP2 exhibited high specificity and sensitivity for the limited number of macaque serum sample we wanted to test if the same was true for a larger sample size. Hence the binding of the peptide OP2 with BV+ and BV- rhesus and cynomolgus sera was measured in a direct binding assay. To determine the total number of BV+ sera identified and BV- sera not identified by OP2 a cut-off of OD ($A_{405-490}$) = 0.3 was used. The sera that on binding with OP2 had OD ($A_{405-490}$) > 0.3 were considered BV+ positive and sera with OD ($A_{405-490}$) < 0.3 were considered negative. Out of the 30 BV+ rhesus and cynomolgus macaque sera tested 28 and 17 respectively, were found to be positive (Fig. 15 a and c). Out of the 20 BV- rhesus and cynomolgus macaque sera tested none of them were found to be positive (Fig. 15 b and d). Hence the sensitivities and specificities of OP2 for BV+ rhesus and cynomolgus sera are 93.3% and 56.7%, and a 100% respectively.

3.9 Testing the cross-reactivity of the peptide OP2 with HSV sera.

To validate that the OP2 does not cross react with HSV sera the peptide was tested with only HSV1+, only HSV2+, both HSV1 and 2 positive, both HSV1 and 2 negative sera in a direct binding ELISA. OP2 did not cross react with either of the HSV1 and 2 standard serum pools. It only reacted with the mAb 9F1, 1 out of 20 HSV1 positive only sera, 4 out of 20 HSV2 positive only sera and 13 out of 20 both HSV1 and 2 positive serum samples (Fig. 16 a-c). Unexpectedly OP2 also non-specifically reacted with 6 out of 20 HSV1 and 2 negative serum samples (Fig. 16d). These results show that that even though OP2 does not cross react with HSV1 + only and HSV2+ only sera the peptide appears to be a lot less specific when being tested with both HSV1 and HSV2 antibodies simultaneously.

3.10 Aim II Conclusions

Zoonotic B virus infection causes rapidly debilitating fatal central and peripheral nervous system disease in humans that is challenging to diagnose early when the presence of cross reactive HSV1 and HSV2 serum and/or CSF antibodies are present [92]. Hence, in our effort to develop an accurate serological assay for unequivocal identification of zoonotic B virus infection, we isolated and characterized a BV-specific reagent, mAb 9F1, to map BV-specific epitopes[96]. To identify the amino acid residues critical for the epitope function, we used a novel combination approach comprising random peptide phage display, overlapping peptide arrays, and compound mutagenesis.

Through phage display epitope mapping we identified an epitope comprised of an “RPGD” motif, which mapped precisely to aa 461-465 region of the possibly one of the most surface accessible regions of the predicted domain II of BV gB protein (Fig. 5B). With this information, we synthesized OP2 Mod peptide, a 12-aa peptide, BVgB sequence aa 457-468 with the

“RPGD” motif at its center with activity greater than any of the phage display peptides when using a direct binding ELISA. The overlapping peptide array analysis revealed the complete epitope and the constitutive aa residues that were used to synthesize four peptide sequences of high specific activities. Because overlapping peptide array epitope mapping is a semi quantitative method, additional testing of the activity of the mAb reactive peptides using peptide ELISA was done in combination with compound mutagenesis to define and refine immunoreactive regions of identified immunoreactive epitopes, or mimotopes [96].

The multiple amino acid modifications made within domains flanking the core consensus region of the BV- specific epitope we study here facilitated identification of a collective effect of the residues on specificity without synthesizing custom phage display libraries that are generally employed for fine epitope mapping [97-100]. We determined that for full activity the consensus sequence “ERRPGD” alone is insufficient in that the immunoreactivity of the peptide size was improved with 15 aa, “ER”, “RPGD” and “AAA” motifs at the N terminus, center and at the C terminus respectively. The optimal size of 15 aa probably represents the epitope size that can be accommodated in the antigen binding pocket of the mAb. Other methods involving mutagenesis of the antigen fragment representing the putative epitope displayed on yeast or phages do not validate the specificity of the mAb selected phages. Hence analyzing the specific activity of the peptides expressed on these cells/phages may provide erroneous data regarding the role of individual aa residues in epitope function[101-104].

The comparison of the relative activities of overlapping peptides (Fig. 4), each individually representing the actual epitope sequence, suggests that either of the four overlapping peptides OP1, OP2, OP3 and OP4 could be used to effectively detect a specific anti-B virus antibody. But using the entire epitope sequence, BVgB aa 451-474, might not be optimal for serological

differential diagnosis of B virus in humans as it might contain cross reactive aa residues. Any conservative replacement or deletions of certain aa residues in some of these OPs negatively affected the activity of these peptides suggesting the functional relevance of the original residues. Out of all the overlapping peptides OP4 might be the best candidate for the serological differential diagnosis of B virus infections as in addition to satisfying all the three aforementioned conditions for maximum activity it also has maximum number of aa acid residues that are unique to BV (Fig. 3D).

The 15-fold increase in the peptide activity of CS Mod1 by addition of alanine to both amino and carboxy terminals of CS peptide sequence could be attributed to a favorable entropy change that might have offset the enthalpy loss and may not necessarily be due to the binding of alanines to the paratope of the 9F1 as described by Greenspan and Di Cera [105]. When comparing CS Mod1 peptide sequence and peptide activity with that of OP3 (Table 2) it is evident that either the aa residues flanking ERRPGDAAA are not involved in binding with the paratope, are neutral, or they produce the same thermodynamic stability as the alanine substitutions of the original aa residues. Despite comparable activity to OPs CS Mod1 may not be a good candidate for differential diagnostics as it still misses the unique aa residues that distinguish it from the corresponding HSV1 and HSV2 epitope.

In direct ELISA it was observed that PhdP3 activity is significantly ($p < 0.0001$) higher than that of other PhdPs (Fig. 4C, D) but in cELISA PhdP3 activity was comparable to other PhdPs (Fig. 2C, E). This might be due to a blocking disadvantage posed by the cyclic nature of the PhdP3 or the differences in the binding opportunities presented to the mAb by the immobilized peptide, in direct ELISA, versus the solubilized peptide in cELISA. The

immobilized peptide might provide a better opportunity to bind to the mAb compared to the solubilized peptide.

The overlapping peptide 2 (OP2) of sequence ELLREQERRPGDAAA was mapped to a region (residues 447-478) of the 3D model of B virus gB. This region corresponds to the predicted disordered segment of domain II of HSV1 gB, the only closely related glycoprotein for which the 3D structure is available and also the most conserved herpesvirus entry glycoprotein [44]. The disordered segment is found on the lateral side of gB and is postulated to be involved in an umbrella like folding and unfolding of gB between pre and post fusion states. An anti-HSV1 mAb C226 that binds to a discontinuous epitope (234-472), an epitope that partially falls in the HSV1 gB segment (464-487), corresponding to our B virus epitope (451-474), can neutralize HSV1[50] suggesting that mAb 9F1 might have a neutralizing function. But the 9F1 failed to neutralize B virus in a plaque reduction neutralization assay (unpublished results). This region has also been shown to be the locus of post-translational modification in some herpesviruses[106]. This might suggest that the blocking of this epitope using the mAb 9F1 might hinder the post-translational modifications, as the protein is being released from the golgi of the host cell, and thus may result in the loss or alteration of gB's activity, which could be its interaction with gD or gH/gL complex, in addition to the fusion of the virus with the host cell [107-109].

3.11 Tables

Table 5. Peptide sequences of the selected phage clones.

Phage Clone	Amino acid sequence																		
7M-1	S W R P G E Y G G G																		
7M-2	S W R P G D Y G G G																		
7M-3	S W R P G D Y G G G																		
7M-4	S W R P G D Y G G G																		
7M-5	S W R P G D Y G G G																		
7M-6	S W R P G D Y G G G																		
7M-7	S W R P G D Y G G G																		
7M-8	S W R P G D Y G G G																		
7M-9	S W R P G D Y G G G																		
7M-13	S W R P G D Y G G G																		
7M-14	S W R P G D Y G G G																		
7M-15	S W R P G D Y G G G																		
7M-16	S W R P G D Y G G G																		
7M-17	S W R P G D Y G G G																		
7M-18	S W R P G D Y G G G																		
7M-19	S W R P G D Y G G G																		
7M-20	S W R P G D Y G G G																		
Cyc-1	A	C	W	R P G	D	M	A	C	G	G	G								
Cyc-4	A	C	W	R P G	D	M	A	C	G	G	G								
Cyc-5	A	C	W	R P G	D	M	A	C	G	G	G								
Cyc-9	A	C	W	R P G	D	M	A	C	G	G	G								
Cyc-10	A	C	W	R P G	D	M	A	C	G	G	G								
Cyc-14	A	C	W	R P G	D	M	A	C	G	G	G								
Cyc-15	A	C	W	R P G	D	M	A	C	G	G	G								
Cyc-16	A	C	W	R P G	D	M	A	C	G	G	G								
Cyc-19	A	C	W	R P G	D	M	A	C	G	G	G								
Cyc-3	A	C	T	P	R P D G	R	C	G	G	G									
Cyc-7	A	C	D	D	R	R P G	S	C	G	G	G								
Cyc-8	A	C	D	L	I	R	R P G	L	C	G	G								
Cyc-6	A	C	S	L	R P G	D	L	C	G	G	G								
12M-1	A	N	W	S	H	W	E	F	N	R	R P G	G	G						
12M-2	A	N	W	S	H	W	E	F	N	R	R P G	G	G						
12M-8	A	N	W	S	H	W	E	F	N	R	R P G	G	G						
12M-12	A	N	W	S	H	W	E	F	N	R	R P G	G	G						
12M-13	A	N	W	S	H	W	E	F	N	R	R P G	G	G						
12M-14	A	N	W	S	H	W	E	F	N	R	R P G	G	G						
12M-18	A	N	W	S	H	W	E	F	N	R	R P G	G	G						
12M-5	G	N	W	C	H	R	E	D	N	R	R P G	G	G						
12M-7	G	N	G	C	H	R	G	D	N	R	R P G	G	G						
12M-3	S	W	F	N	D	E	L	D	R	R P G	G	G	G						
12M-4	A	G	W	S	P	P	G	F	D	R	R P G	G	G						
12M-9	R	N	D	P	F	A	W	K	D	R	R P G	G	G						
12M-11	G	H	T	A	V	S	G	D	D	R	R P G	G	G						
12M-15	T	F	S	N	F	G	S	N	D	R	R P G	G	G						
12M-17	S	I	D	R	Y	P	G	W	R	R S P	G	G	G						
12M-6	G	F	T	V	F	T	S	H	D	C	R P G	G	G						
12M-19	M	N	D	P	F	A	W	K	D	C	R P G	G	G						
Consensus											R P G								
BV gB (452-471)	V	R	E	L	L	R	E	Q	E	R	R P G D	A	A	A	T	P	K		

7M, 7-mer phage; Cyc, loop-constrained heptapeptide cyclic phage; 12M, 12-mer phage; BV gB, B virus glycoprotein B. The motifs common in all the sequences are highlighted in bold. Overall consensus is highlighted on a segment (residues V452- K471) of BV gB sequence in bold.

Table 6. Summary of characteristics and activity of the synthesized peptides.

Core Motif	Supplementary Motifs	Name	No. of AA	Amino acid sequence	Peptide Activity
Motif R PGD	AAA and ER present	OP2	15aa	ELLREQERRPGDAAA	4.58
		OP3	15aa	REQERRPGDAAATPK	4.42
		OP4	15aa	ERRPGDAAATPKPSA	4.32
		CS Mod1	15aa	AAAERRPGDAAAAAA	4.38
		OP2 Mod1	12aa	REQERRPGDAAA	4.35
	AAA missing	OP1	15aa	YVRELLREQERRPGD	3.97
		OP1 Mod	15aa	FLHDVVREQERRPGD	2.58
		OP2 Mod2	9aa	REQERRPGD	0.27
		CS	6aa	ERRPGD	0.29
	ER missing	CS Mod2	15aa	AAAAARRPGDAAAAAA	0.24
		CS Mod3	15aa	AAAAAAAARRPGDAAA	0.22
	ER and AAA missing	PhdP3	12aa	CWRPGDMACGGG	4.24
		PhdP2	10aa	SWRPGDYGGG	1.07
Motif R PG	ER and AAA missing	PhdP1	15aa	ANWSHWEFNRRPGGG	0.18
None	ER and AAA missing	No Peptide	15aa	----	0.26

Aa, Amino acids; mAb, Monoclonal antibody; Mod, modified; OP, Overlapping peptide; CS, Core sequence; PhdP, Phage display peptide. The motifs present on the BV gB sequence are highlighted in bold. Peptide activities shown are the mean values of absorbance (A405-490nm) of peptides in triplicates at 1:6520 dilution of mAb 9F1.

3.12 Figures

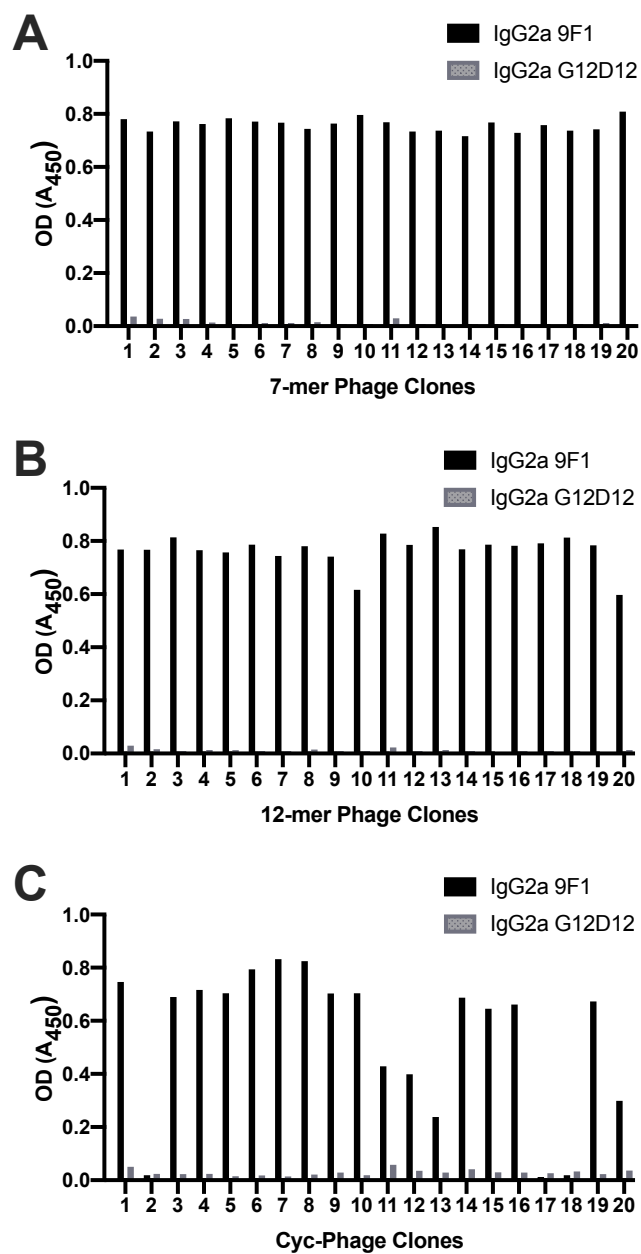


Figure 8. Immunoreactivity of selected phage clones expressing random peptides with mAb 9F1 in a phage enzyme-linked immunosorbent assay (pELISA).

Twenty phage clones with (a) 7-mer, (b) 12-mer, and (c) random cyclic peptide displays were randomly selected after three rounds of biopanning and detected using mAb 9F1 and G12D12 (negative control).

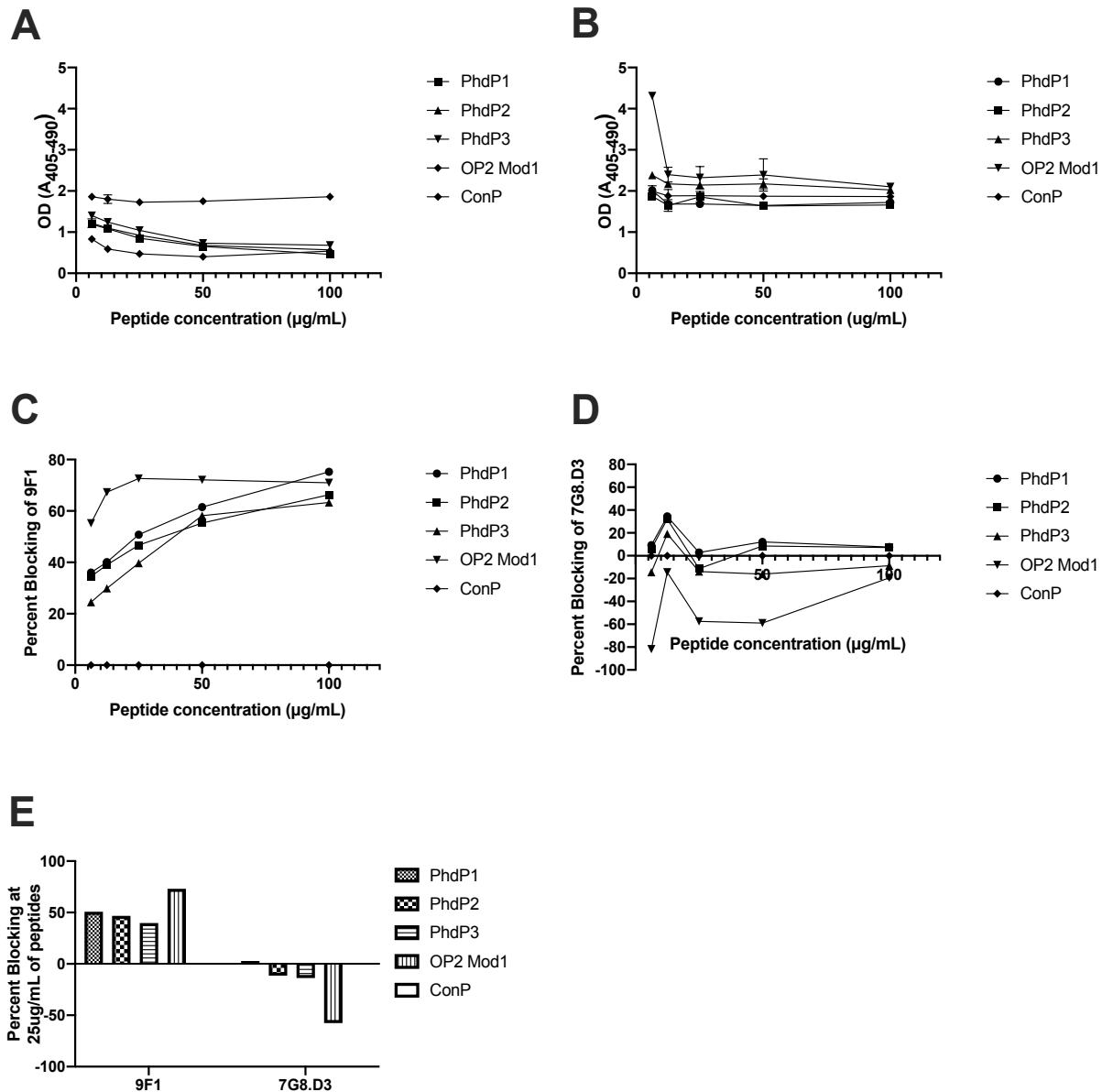


Figure 9. Testing if the peptides represent or mimic the 9F1 epitope by measuring the blocking of BV-specific mAb 9F1 binding to BV whole antigen by the peptides.

Blocking of (a) mAb 9F1 (b) mAb 7G8 binding to BV whole antigen by the synthetic peptides.

The percent blocking of (c) the mAb 9F1 and (d) control mAb 7G8 binding by the peptides calculated using the formula $100 - (\text{OD of the peptide} / \text{OD control peptide (ConP)}) \times 100$.

(e) Percent blocking of mAb 9F1 and mAb 7G8.D3 binding by the peptides at 25µg of peptides/mL.

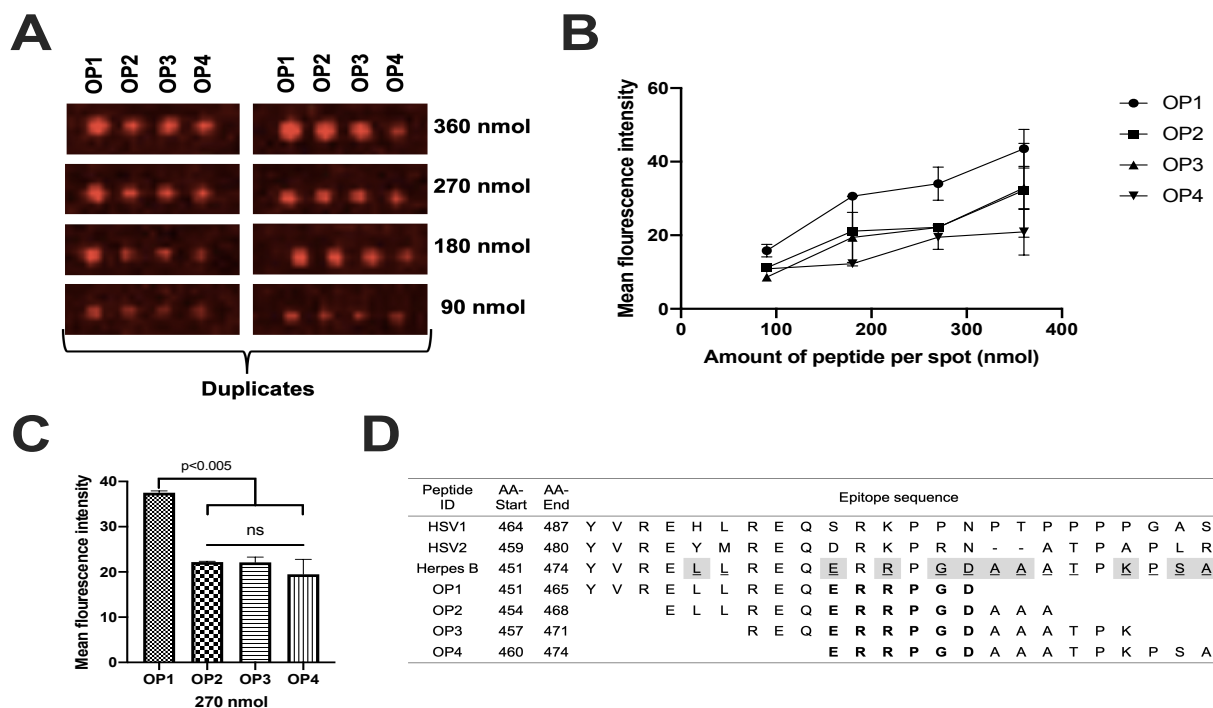


Figure 10. Identification of epitopes of mAb 9F1 using overlapping peptide array.

An overlapping peptide array of BV gB amino acid sequence with 15 amino acids long peptides and an overlap of 4 amino acids between the peptides was used. (a) A cropped section of the peptide array slide showing only the positive spots. The observed fluorescence is due to the binding of mAb 9F1 with the four overlapping peptides (OP1-4) that were coated at three fold dilutions from 460- 90 nmol/spot in duplicates. (b) The mean fluorescence intensities were calculated for each overlapping peptide at different concentrations using ImageJ. (c) Comparison of the mean fluorescence intensities of OPs (1-4) at the concentration of 270 nmol/spot. (d) The aa sequence of 9F1 epitope of herpes B virus gB (UniProtKB - Q7T5E1), the corresponding epitopes of HSV2 (UniProtKB - P08666) and HSV1 (UniProtKB - P10211), the overlapping peptides and their location on the BV gB sequence is shown. The aa residues that are unique but are common to either HSV1 or HSV2 are underlined, aa residues that are unique only to herpes B virus are underlined and highlighted in grey. Consensus sequence of overlapping peptides is highlighted in bold.

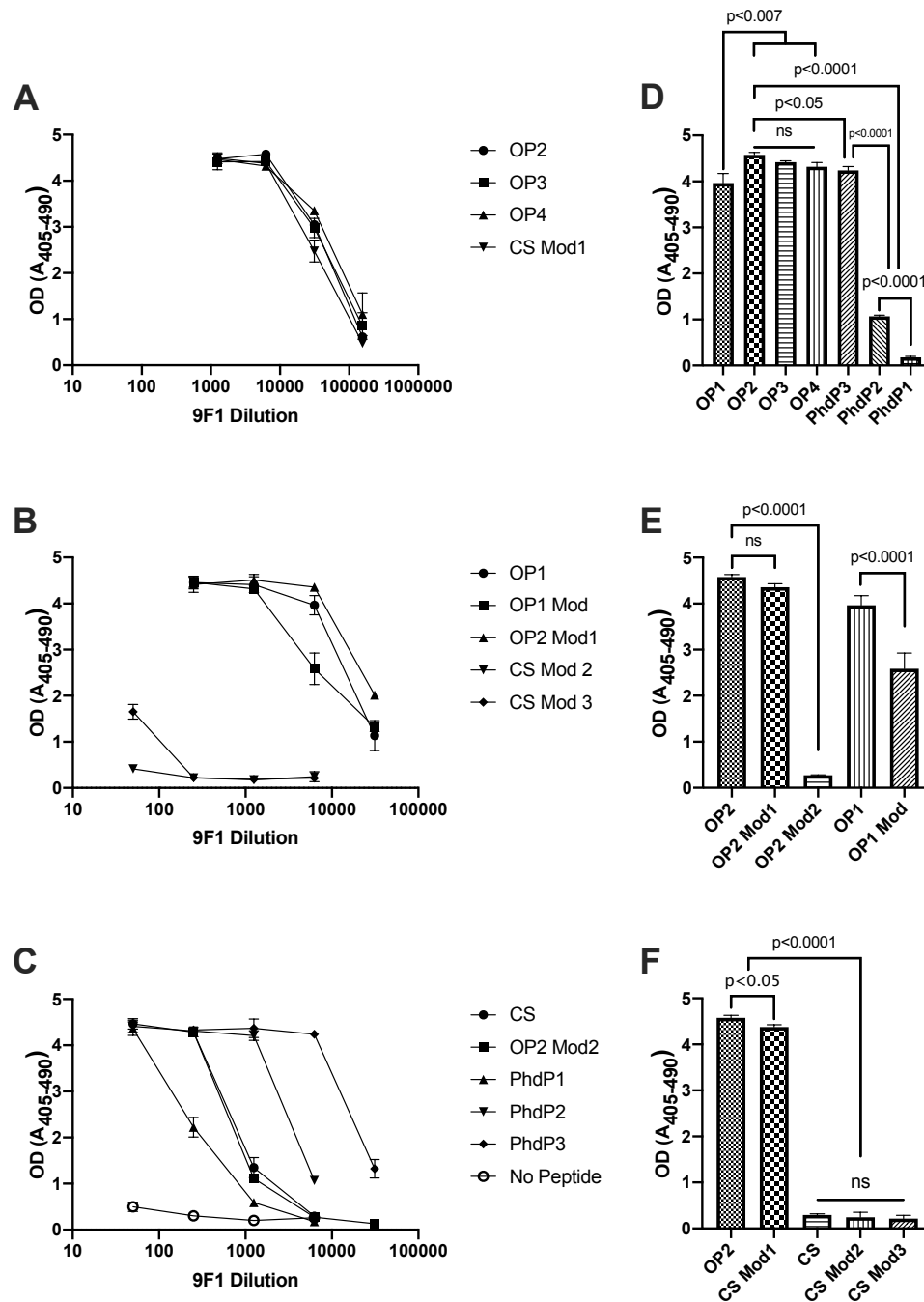


Figure 11. Measuring the peptide activity in a direct binding assay

The synthesized biotinylated peptides were tested for their binding to the mAb 9F1 by using the peptides as an antigen in a direct ELISA. The OD values represent average mean values from triplicate tests. The peptide binding with mAb 9F1 was grouped based on the amino acid residues present in the peptides (a) Peptides with “RPGD”, “ER”, “AAA” motifs and are 15 aa long, (b)

peptides missing either one of these elements and (c) peptides missing two or more of these elements. The binding of peptides to 1:6250 dilution of mAb 9F1 was compared between (d) peptides identified using overlapping peptide array and phage display (e) OP2 and OP1 with their respective modifications. (f) OP2 and peptides synthesized based on the consensus sequences, from peptide array and phage display, and the modifications of the consensus sequence.

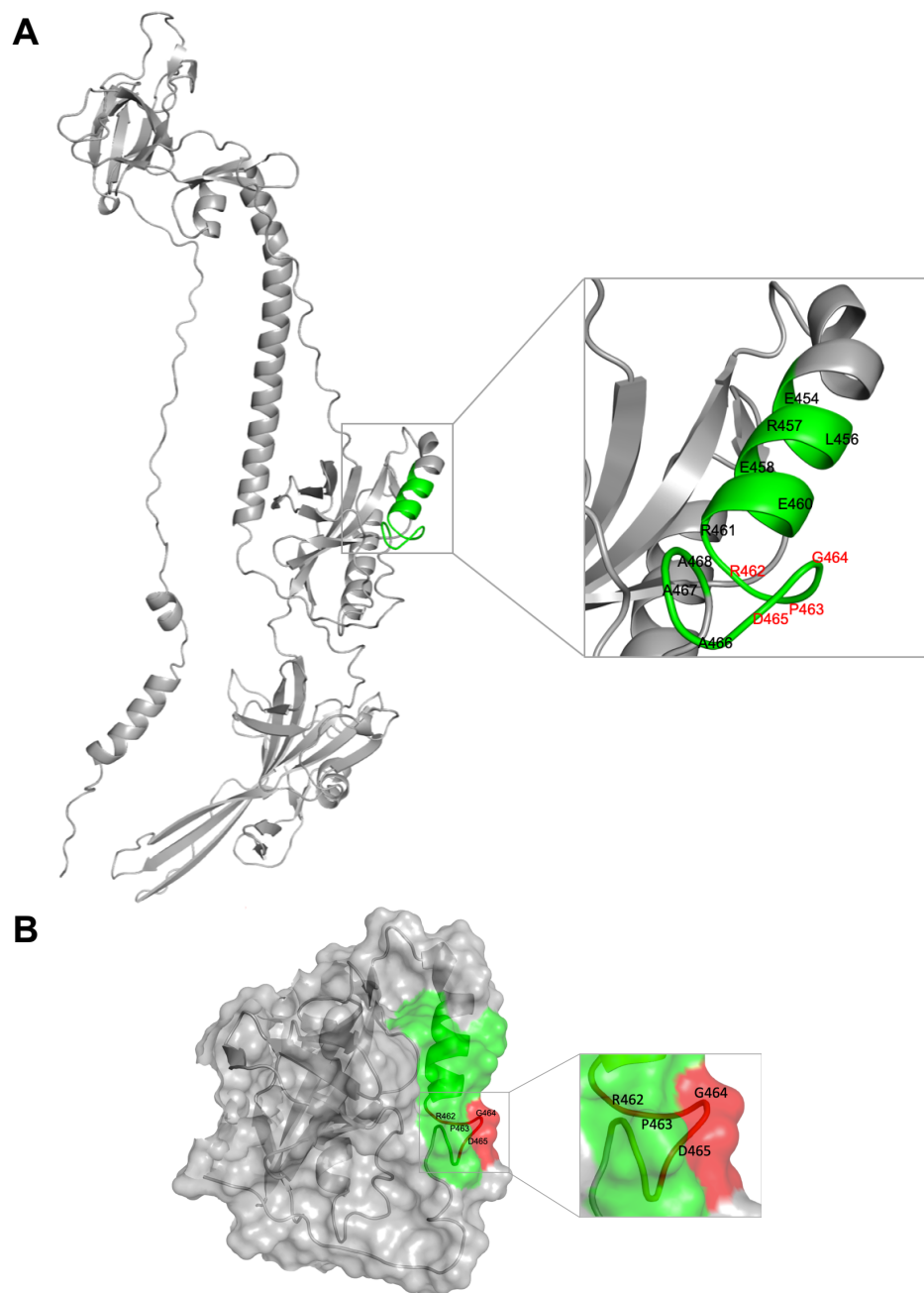


Figure 12. Mapping of the epitope (OP2) ELLREQERRPGDAAA to a three-dimensional model of a BV gB monomer.

A 3D model of the monomer of gB ectodomain (residues G72-E891) was generated by projecting the BV gB sequence on the crystal structure of full length HSV1 gB (PDB code 6BM8). Panel A, ribbon representation of BV gB monomer (residues A98-A712), shows the epitope (OP2) ELLREQERRPGDAAA (residues 454-468) highlighted in green. The inset shows

the location of individual amino acid residues that constitute the epitope. Panel B, a solvent-accessible surface representation of the predicted domain II (residues 129-140, 351-446, 447-478) of BV gB, shows the location of the complete epitope (green) and consensus sequence RPGD (red) on domain II. The consensus sequence RPGD is magnified in the inset to emphasize the surface accessibility of the residues.

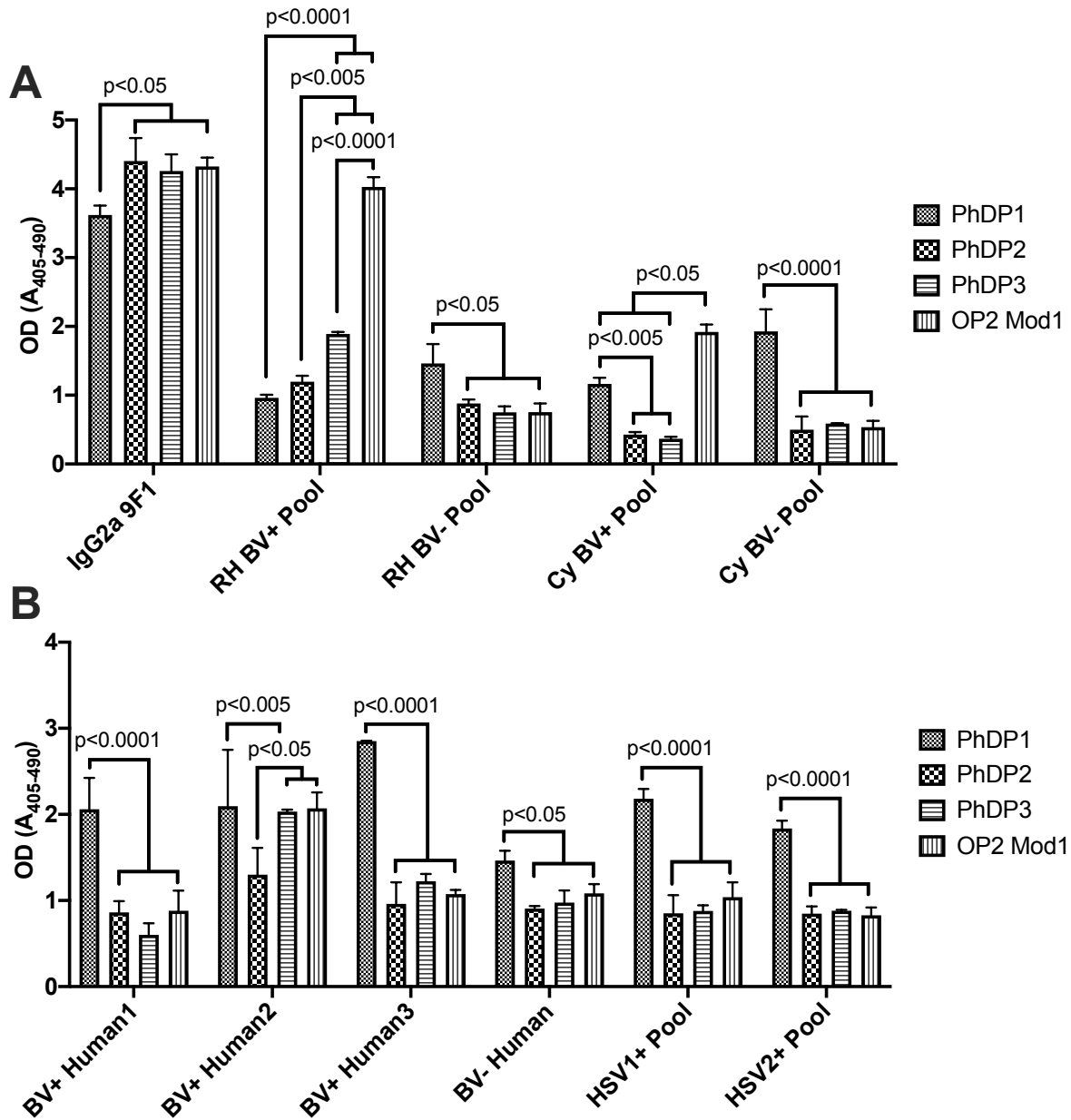


Figure 13. Testing the specificities of the peptides, identified by phage display in a direct binding enzyme linked immunosorbent assay.

The specificities of PhdPs 1-3 and OP2 Mod1 (discovered from alignment of PhDPs consensus sequence with BV gB) for **(a)** Rhesus, cynomolgus and **(b)** human sera are shown as absorbance values at 409-490nm .

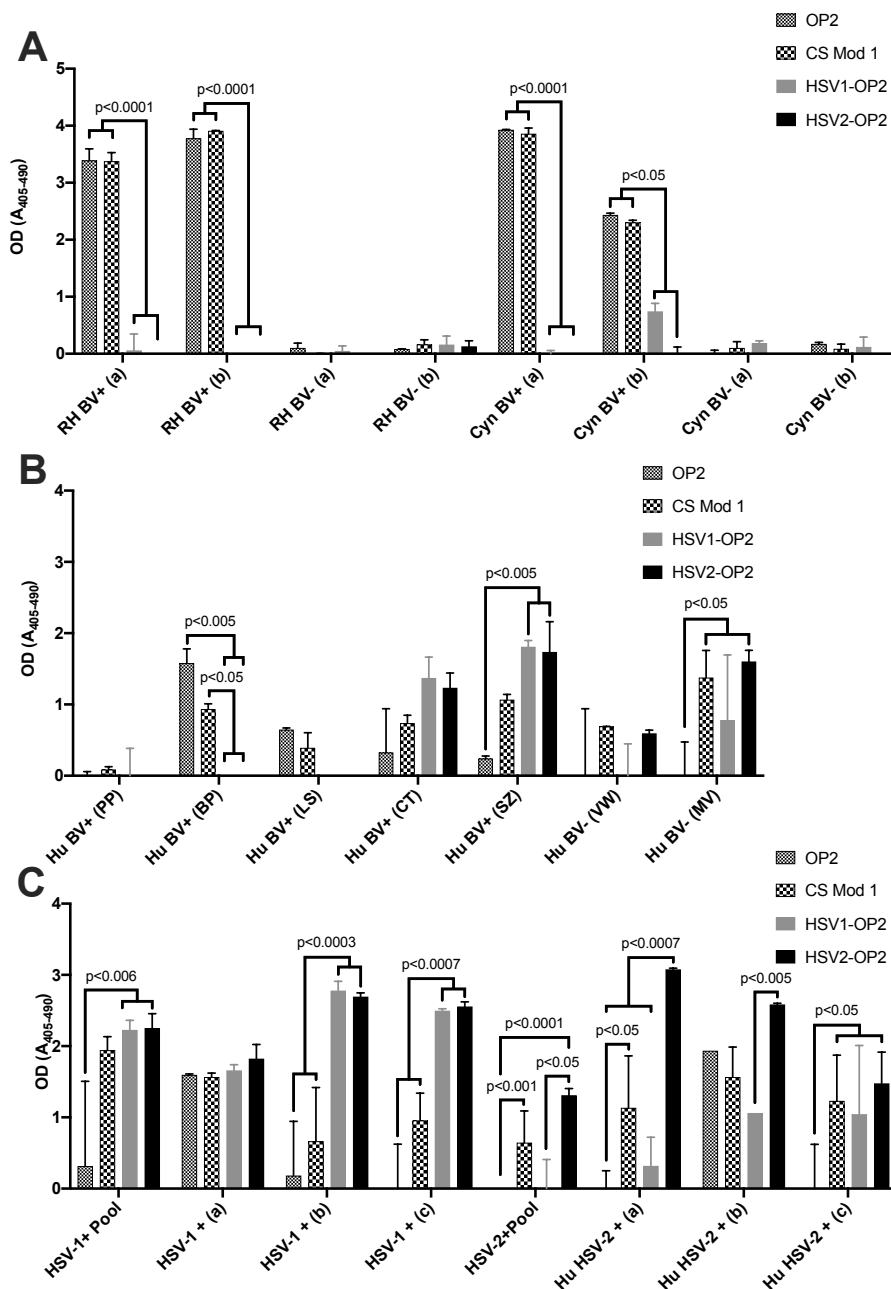


Figure 14. Testing the specificities of the peptides, identified by peptide array and compound mutagenesis studies, in a direct binding enzyme linked immunosorbent assay.

The specificities of OP2, CS Mod1, HSV1-OP2 and HSV2-OP2 peptides for (a) Rhesus, cynomolgus and (b) human BV positive and negative sera, and (c) human HSV sera are shown as absorbance values at 409-490nm.

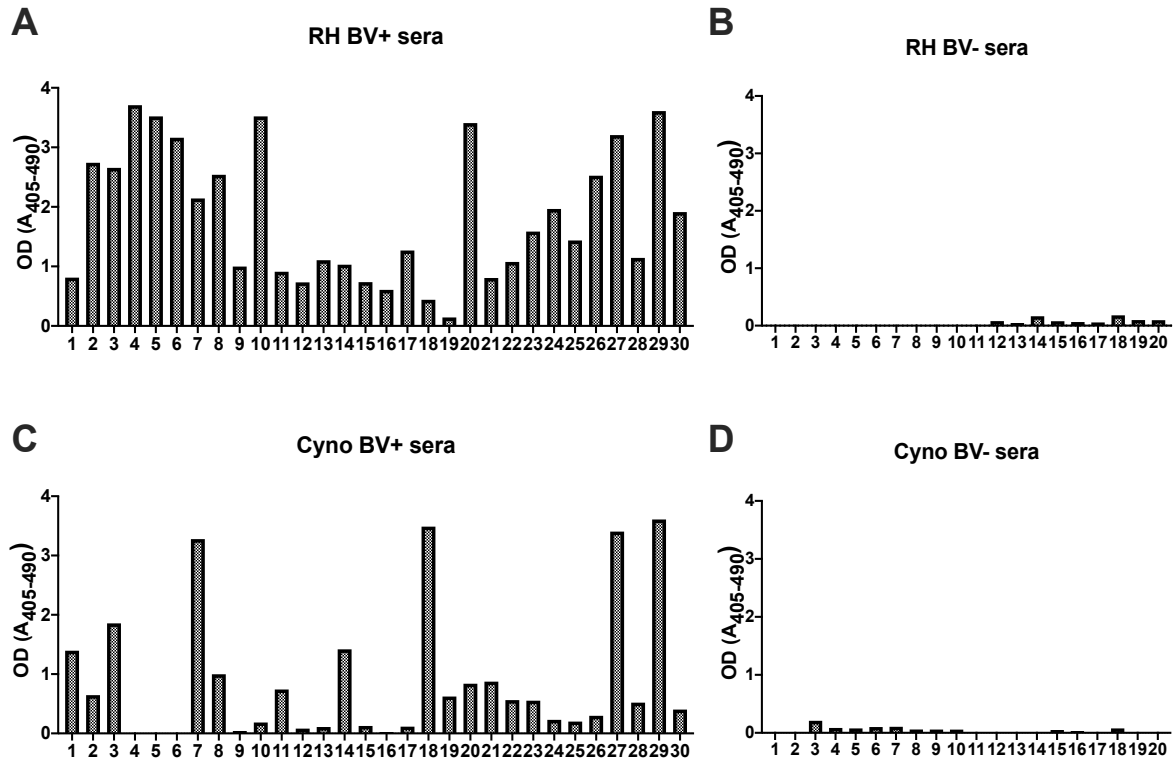


Figure 15. Determining the sensitivity and the specificity of OP2 peptide for

Macaque sera.

The peptide OP2, identified using peptide array, was tested for its sensitivity and specificity for the BV infected rhesus and cynomolgus macaques. ELISA plates were coated with OP2 and the binding of polyclonal antibodies from **(a)** BV infected rhesus sera, **(b)** BV uninfected rhesus sera, **(c)** BV infected Cynomolgus sera, **(d)** BV uninfected cynomolgus sera was determined by measuring the OD (absorbance at 405-490nm).

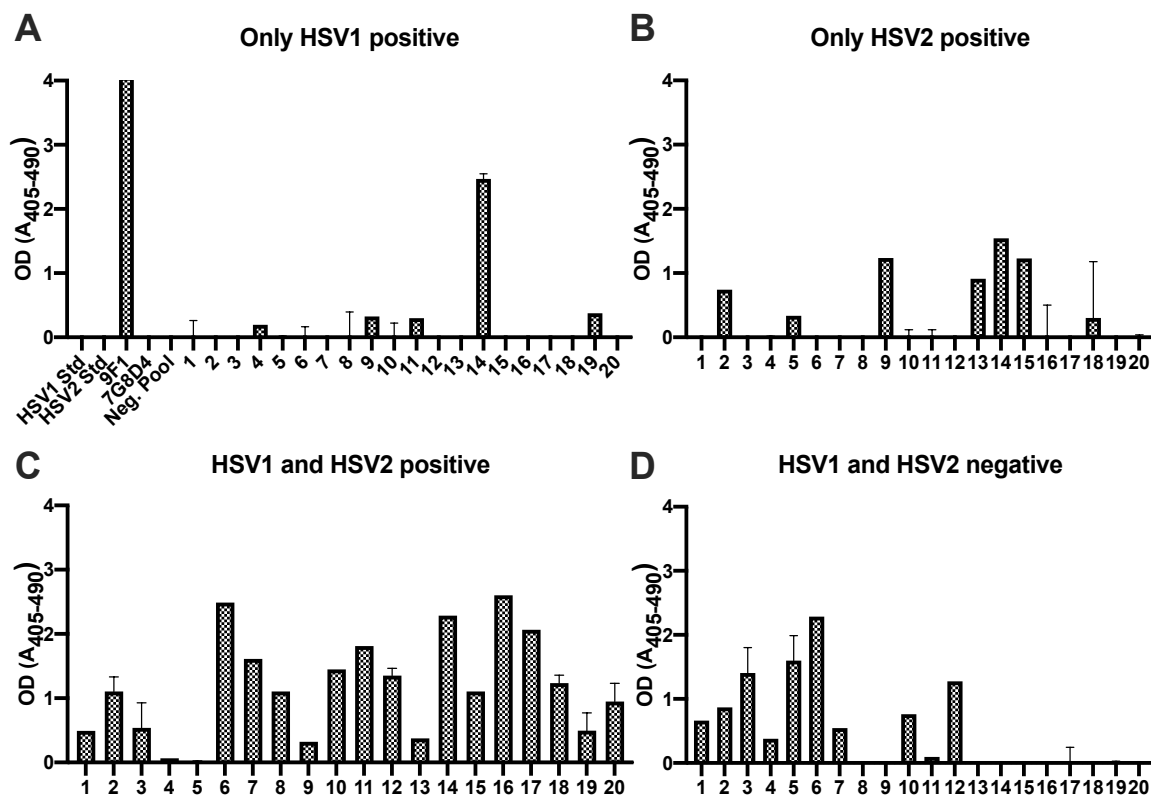


Figure 16. Testing the cross-reactivity of the peptide OP2 with HSV1 sera.

The cross-reactivity of the peptide OP2 with sera from (a) HSV1 only positive humans, (b) HSV2 only positive humans (c) both HSV1 and HSV2 positive humans (d) both HSV1 and HSV2 negative humans was measured in a direct binding ELISA by determining the binding of sera to the coated peptide by measuring the OD (absorbance at 405-490nm).

4 MATERIALS AND METHODS

4.1 Epitope mapping by commercial random peptide phage display library

Mapping of BV gB epitopes specific for monoclonal antibody IgG2a 9F1 was performed using linear random peptide phage display libraries (heptapeptide: Ph.D.-7, dodecapeptide: Ph.D.-12 and loop-constrained heptapeptide: Ph.D.-C7C; New England Biolabs) as per manufacturer's instructions. Briefly, the wells of flat bottom Maxisorp immunoplates (Nunc) were coated with 150 μ L of 100 μ g/ml 9F1 mAb/well in 0.1 M NaHCO₃ buffer (pH 8.6) and incubated overnight at 4°C with gentle agitation in a humidified container. The coating solution was poured out and 300 μ L of blocking buffer (5mg/mL bovine serum albumin in 0.1 M NaHCO₃; pH 8.6) was added, followed by incubation at 37°C for 1 h. After washing the plate 6x with TBST (TBS + 0.1% (v/v) Tween-20), 2x10¹¹ phage clones diluted in 100 μ L of TBST were added and incubated for 1 h at 37°C with gentle agitation. After washing the plate 10x with TBST the bound phages were eluted with 100 μ L of 0.2 M Glycine-HCl (pH 2.2) for 10 min and neutralized with 15 μ L of 1 M Tris-HCl (pH 9.1). The eluted phages were amplified in ER2738 culture and precipitated with 20% PEG 8000/2.5 M NaCl. After one round of negative selection using mAb G12D122 two additional rounds of selection of amplified phages, twenty random well separated plaques were selected, amplified and tested by ELISA.

Phage Titration

To each 10 μ L of ten-fold serially diluted phages 200 μ L of a mid-log phase ER2738 culture was added and incubated at RT for 2 mins. Each phage dilution was then mixed with 3 mL of 45°C top agar and then poured onto a pre-warmed LB/IPTG/Xgal plate. The plates were allowed to cool for 5 minutes and incubated overnight at 37°C. The plaques were counted on

plates that had approximately 100 plaques.

4.2 Phage ELISA I (Phage capture by coated mAb)

Each of the 20 blue plaques picked from the titration plate were transferred to tubes containing 1 ml of 1:100 diluted overnight culture of ER2738. The tubes were incubated at 37°C with shaking for 5 hours. The bacteria were pelleted down and 5 µl of the supernatant from each clone was added to a 20 ml culture for each clone and incubated with vigorous aeration for 5 hours at 37°C. The bacteria were pelleted down and the phage was precipitated from the supernatant using 20% PEG 8000/2 .5 M NaCl. An ELISA plate was coated with 100 µg/ml of target mAb in 0.1 M NaHCO₃, pH 8.6 and incubated overnight at 4°C in a humidified chamber. The plates were then incubated with blotto for 1 hour at 37°C. The phage clones (10¹¹ virions) were diluted 1:10 in blotto and incubated with shaking at RT for 2 hours. HRP-anti M13 (GE Healthcare, USA) diluted at 1:5000 in blotto was added and incubated at 37°C for 1 hour. The plates were washed 6x with TBST after each incubation. TMB substrate (ThermoFisher, USA) 100ul/well was added and the plates were incubated at 37°C for 20 min. To stop the reaction 1M H₂SO₄ 50ul/well was added and the absorbance (OD_{450nm}) was measured using a plate reader.

The positive phage clones were sequenced, and the peptide sequences were synthesized commercially (Peptide 2.0, Chantilly, VA). An additional lysine was added to the C terminal of certain peptides for biotinylation.

4.3 Phage ELISA II (Antibody capture by coated phage)

An ELISA plate was coated with 10¹¹ PFU/mL phages in 0.1 M NaHCO₃, pH 8.6 and incubated overnight at 4°C in a humidified chamber with shaking. The plates were then

incubated with blotto for 1 hour at 37°C. The mAbs were diluted two folds from 40 µg/mL-2.5µg/mL in blotto and incubated at 37°C for 1 hours. Anti-mouse IgG H+L (Bethyl Laboratories, USA) alkaline phosphatase conjugated secondary antibody diluted at 1:500 in blotto was added and incubated at 37°C for 1 hour. The plates were washed 3x with BBST after each incubation. To prepare the substrate solution p-nitrophenyl phosphate (pNPP) tablets (Sigma) were added in 1 M diethanolamine buffer containing 0.5 mM MgCl₂, pH 9.8 (1 mg of pNPP/ml), and 200 µL of freshly prepared mixture was added to each well. The reaction was stopped by adding 50µL of 3N NaOH per well after 30 minutes of incubation and the optical densities (ODs) were read on an automatic microplate reader at 405-490 nm.

4.4 Epitope mapping by fd random peptide phage display library

The fd phage display library (linear and looped 8,6,10,12-mer peptides) were a kind gift from Dr. Jonathan Gershoni, Tel Aviv University, Israel. For the first round of positive selection the mAb 5A2 (10µg) was mixed with 1×10^{10} phage library in a total volume of 100 µl of blocking solution (3% BSA in TBS. After incubating the tube on a shaker for 1 hour at RT 50µl of Invitrogen Protein G Magnetic Beads were added to the tube and incubated for another 30 minutes at RT on a rotator. The beads were washed three times with TBST. The bound phages were eluted using 105µl of 0.1M glycine pH2.2. To neutralize the pH 19µL of TRIS-HCl pH 9.1 was added. The eluted phages were titrated and used to infect an exponentially growing DHF5α F+ culture with intact pilli. Infected bacteria were allowed to amplify in 100 ml volume of 2YT medium, in 0.5 litter flask, at 37° C, at 225 rpm, for 20 hours. Tetracycline (20 µg/ml final concentration) was added after one hour of incubation. After pelleting the bacteria, the phages were precipitated out using 33% PEG 6000/3.3 M NaCl to obtain the amplified bound phages.

A negative selection round was performed using the bound phages from the first round of selection using the same steps as before except a control mAb 7G8 was incubated with the phages. The unbound phages were amplified and used for two more rounds of positive selection.

Titration of the phages

The phages to be titrated were diluted 10 folds. An overnight DH5 α F+ (200 μ L) culture was mixed with 3.5 mL of 0.5% agarose and immediately pour over a pre-warmed LB-agar plate. After cooling, a drop (2 μ l) of each phage dilution was applied to two squares (duplicates) marked on the LB-agar plate. The drops were allowed to dry and incubated overnight 37 °C. The number of plaques in each square were counted under a microscope and an average was calculated.

4.5 Epitope mapping by peptide array

Overlapping peptide array was performed in collaboration with Ulrike Beutling, Dr. Mark Broenstrup from the Department of Chemical Biology, Helmholtz Centre for Infection Research, Germany. A peptide array based on the sequence of gB herpes B strain E2490 (GenBank accession number AF533768) was prepared and used as described before for the assay [95].

4.6 Amplification of mAb binding fd phage recombinant pVIII gene

The mAb selected phages were used directly for PCR using for Platinum SuperFi Green PCR Master Mix (Invitrogen, USA) as per the manufacturer's instructions. PCR, performed in triplicates for each sample, was used to amplify the recombinant pVIII gene of the phages. For 15 PCR cycles two consecutive steps of denaturation at 98°C for 2 and 20 min followed by 5 seconds of extension at 72°C were performed. The annealing step was omitted since the T_m of the oligos was very high – approaching the extension temperature. Forward primer

5'ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGGCGGCCAACGTGGC 3' and reverse primer 5' **GACTGGAGTTCAGACGTGTGCTCTTCCGATCTTGGTCGGCCCCA** GCGGC 3' with partial illumina adaptors (highlighted in bold) were used for the PCR.

Purification and quality control of Amplicons

The amplicons were purified using Mag-Bind Total Pure NGS beads (Omega Bio-Tek, USA) as per the manufacturer's instructions. Briefly, the PCR samples were mixed with the beads and incubated for 5 mins. The beads were washed with 70% ethanol twice, air dried and eluted by incubating with 10 mM Tris pH 8.0 for 5 mins. The DNA concentration of the eluates was determined for normalization using Qubit 4 (ThermoFisher, USA) as per the manufacturer's instructions and the size of the amplicon was confirmed by agarose (2%) gel electrophoresis.

4.7 Next Generation Sequencing of 5A2 specific phages

Next generation sequencing (NGS) on the illumina platform was performed by Genewiz corporation. Briefly, PCR amplicon samples were prepared with the TruSeq library preparation kit and sequenced on the MiSeq with 2x250bp configuration. After quality and adapter trimming, paired reads were merged into a single sequence if they overlapped and the overlapped region was identical. The target region was identified by anchoring the highly conserved flanking regions. Consensus sequences were identified and abundances for each consensus sequences were calculated for each sample within the target region between the two highly conserved sequences. After translating the unique nucleotide sequences into protein sequences, a unique protein sequence identification and abundance calculation was performed.

4.8 Competitive Inhibition Binding ELISA of Monoclonal Antibody 9F1 to a Synthetic Peptide

A Competitive Inhibition Binding ELISA (CIBE) was used to determine the specific activity of the peptides. The wells of a flat bottom 96 well plate (Corning, USA) were coated with 1µg of recombinant BV gB diluted in 1X PBS with 0.08% azide and incubated overnight in a humidified chamber at 4°C. The wells were blocked with 150µl of blotto (borate-buffered saline containing 2.5% nonfat dry milk and liquid gelatin)/well for 1 h at 37°C. Blocking and subsequent incubations with peptide-mAb mixture and anti-mouse IgG H+L (Bethyl Laboratories, USA) alkaline phosphatase conjugated secondary antibody were done for 1 h at 37°C. Each incubation step was followed by three washes with borate-buffered saline–0.05% Tween 20. The peptides and monoclonal antibodies were diluted two folds from 100µg/mL – 6.25µg/mL and 1:2000(9F1) and 1:64000 (7G8) respectively in blotto. The peptides were incubated with the specific IgG2a 9F1 mAb and a control mAb IgG2a 7G8 for 1 h at 37°C separately in eppendorf tubes. The peptide-mAb mixture thus obtained and 50µL/ well of each was added to the BV gB coated plate. The conjugate was diluted at 1:500 in blotto, and 50µL/ well was added. To prepare the substrate solution p-nitrophenyl phosphate (pNPP) tablets (Sigma) were dissolved in 1 M diethanolamine buffer containing 0.5 mM MgCl₂, pH 9.8 (1 mg of pNPP/ml). and 200 µL of freshly prepared mixture was added to each well. The reaction was stopped by adding 50µL of 3N NaOH per well after 30 minutes of incubation and the optical densities (ODs) were read on an automatic microplate reader at 405-490 nm. The percent competition was calculated using the formula $100 - (\text{OD of the peptide} / \text{OD control peptide} * 100)$.

4.9 Direct Binding Enzyme-linked immunosorbent assays

The peptide activity was determined by measuring the binding of peptides to human and macaque serum, and mAb 9F1 in a direct ELISA. The wells of a flat bottom 96 well plate (Corning, USA) were coated with 50 μ L of 25 μ g/mL of Streptavidin (New England Biolabs) diluted in 1X PBS with 0.08% azide/well. The plate was incubated overnight in a humidified chamber at 4°C. The wells were blocked with 150 μ L of 2% bovine serum albumin in BBS (borate-buffered saline)/well. Blocking and subsequent incubations with peptides, serum, IgG2a 9F1 mAb and anti-mouse IgG H+L (Bethyl Laboratories, USA) alkaline phosphatase conjugated secondary antibody were done for 1 h at 37°C. Each incubation step was followed by three washes with borate-buffered saline–0.05% Tween 20. The biotinylated peptides, serum, IgG2a 9F1 and the conjugate were diluted in the blocking buffer at 0.5 μ g/mL, 1:10, five-fold serial dilutions from 1:50 to 1:156250 and 1:500, respectively. To prepare the substrate solution p-nitrophenyl phosphate (pNPP) tablets (Sigma) were added in 1 M diethanolamine buffer containing 0.5 mM MgCl₂, pH 9.8 (1 mg of pNPP/ml), and 200 μ L of freshly prepared mixture was added to each well. The reaction was stopped by adding 50 μ L of 3N NaOH per well after 30 minutes of incubation and the absorbance was read on an automatic microplate reader at 405-490 nm.

4.10 Direct Binding Covalently-linked immunosorbent assays

The peptide activity was determined by covalently linking the peptides via their carboxylic group to the CovaLink (ThermoFisher, USA). The wells of a CovaLink plated were coated with peptides that were first diluted at 70 μ g/mL in sulfo-NHS and then diluted two folds from 20 μ g/mL-1.25, μ g/mL in water. EDC (50 μ L) was added to all the wells and the plates were incubated O/N at RT. The plates were washed with 3 X COVA Buffer and for the last wash

incubated 15 min at RT before discarding followed by blocking using 2% BSA in BBS for 1 h at RT. The primary antibodies diluted 1:100 in blocking buffer were added and plate incubated at RT for 1hr followed by a 5x wash with BBST. Anti-Mouse (H+L) AP diluted 1:500 in blocking buffer was added and the plate was incubated at RT for 1hr followed by 5x wash with BBST. PNPP substrate 200uL/well was added and the plates were incubated at RT for 30 mins and the absorbance was read on an automatic microplate reader at 405-490 nm.

4.11 Mapping the epitopes on 3D model

The sequence of B virus (BV) gB retrieved from uniprot with id Q7T5E1 was used to prepare a model of a hypothetical complex. The known structure of Herpes Simplex Virus (HSV1) gB (PDB code 6BM8) were used as templates for computer modeling of the B virus (BV) gB.

The model structures were prepared using I-TASSER (Iterative Threading ASSEmbly Refinement) software approbated in the framework of Critical Assessment of Structure Prediction (CASP) conferences as described previously[110]. The stability of the B virus (BV) gB protein globule structure was calculated using the package QMEAN (Qualitative Model Energy Analysis)[111]. The conformational epitopes were identified using the webtool Episearch [90]. The sequence alignments were performed using Megalign pro. The illustrations of protein structures were prepared using Pymol [112].

5 CONCLUSIONS

5.1 Novel epitope-focused recombinant protein-based potential BV vaccine candidates

Treatment of zoonotic B virus has been restricted to a few nucleoside-inhibitors, and beyond these interventions options have not changed in nearly 50 years. Hence this study, provides important information to formulate interventions against BV. This study has identified, for the first time multiple gB mimotopes that, individually, represent at least the partial epitope(s) critical for antibody neutralization of the virus, providing for the first time possible novel vaccine candidates that might be used as a therapeutic intervention for late-stage B virus infection. Five out of six mimotope sequences (specially P.4E11.PhD CEPFWTNCGGGS) specific for mAb 5A2 were mapped to the discontinuous epitope (aa residues 113-120 and 516-526) of domain III (or the core) of the BV gB. This is consistent with the targets of neutralizing antibodies as most of them are found buried in the core of the target proteins.

The peptides representing these epitopes should be tested further for their activity. In this work each peptide was tested individually for its binding with the mAb 5A2 but maybe using the six peptides together might result in an improved collective function, as each peptide might represent only a part of the epitope and a combination might represent the complete epitope. Furthermore, their activities should also be tested by measuring their ability to block the neutralization of BV by the mAbs 5A2 and 4E11 in a plaque reduction neutralization assay. If they are able to block the neutralization, they can be used to vaccinate macaques. The macaques can then be monitored for the production of the BV neutralizing antibodies specific for these peptides. If these experiments are successful, a prophylactic, effective and safe intervention against BV infection will be made available. The data from this research will provide physicians and scientists new options for vaccine development for the first time in nearly a half of a century.

5.2 Advantages of Epitope based Vaccines

Although the epitope vaccines are not commercially available yet, the epitope vaccines in addition to being capable of generating specific immune responses have multiple advantages [113]. The epitope-based vaccines can help “focus the immune response”. When a pathogen invades the host, the immune system is exposed to a plethora of antigens. Not all of these antigens will be responsible for a protective function. Moreover, the host cannot generate an equally strong response to all these antigens. Hence by using epitope focused vaccines we can “focus the immune system” to produce a desired set of antibody repertoire. Successful pathogens over multiple rounds of replication inside their hosts are able to distract the immune system by directing it to immunodominant epitopes with no protective value, hence exhausting the immune system. The highly immunogenic mutation prone five variable loops of HIV-1 gp120 constantly exhaust the immune system without producing any protective effect [114, 115]. The epitope-based vaccine could “enhance the immunity” as the selected epitopes can induce the generation of protective antibodies instead of surface reacting antibodies. Epitope based vaccines can also help avoid enhancement of infections caused by Fc-receptor-mediated endocytosis of immunocomplexed pathogens as observed in case of HIV and Dengue infections. Another application of the epitope-based vaccines could be to prevent the generation of auto antibodies. When designing the vaccine, we can blast the epitope sequence to check for any aa residues that might mimic the self-antigens. Any residues that resemble the self-antigens can be excluded from the vaccine to avoid triggering an autoimmune disease.

5.3 Novel marker for differential diagnosis of B virus

This study for the first time describes the use of an epitope-based BV specific peptide that can be used to detect BV specific antibodies and distinguish them from HSV antibodies. By

employing pIII fused random peptide phage display libraries, compound mutagenesis and peptide array we were able to identify a highly reactive epitope of the BV type specific non-HSV cross-reacting mAb 9F1. The peptides corresponding to the phage display sequences and the peptide array sequences were compared for their specificities and sensitivities and it was observed that the peptide OP2 (ELLREQERRPGDAAA) was highly sensitive and specific in detecting BV antibodies from rhesus and cynomolgus macaques. OP2 was also very specific in detecting BV specific antibodies and had low cross reactivity with HSV antibodies but was comparatively less sensitive in detecting the antibodies from human sera.

It was observed that OP2 is specific for BV antibodies and HSV1-OP2 and HSV2-OP2 were specific for the anti-HSV1 antibodies (Fig. 15). The two peptides could be used in conjunction to quantify the amount of cross-reactive antibodies in the serum sample of the suspected BV infected patient to rule out the false positive due to the presence of cross reactive HSV antibodies. Collectively, these results suggest that the peptide OP2 can be used alone or in combination with other BV specific antigens to detect BV specific antibodies in a peptide ELISA. The data from this research will provide physicians and scientists new options for unambiguous diagnosis for the first time in nearly a half of a century.

5.4 A novel, simplified and cost-effective fine epitope mapping approach

We propose the use of random peptide phage display in conjunction with compound mutagenesis as a relatively inexpensive, fast and reliable alternative to other expensive/lengthy approaches that either involve a coating slides with the entire sequence of the target protein as overlapping peptides, or the developing custom phage display libraries in combination with mutagenesis scanning to identify functional epitopes [101-104] . Through this study we propose the following novel approach. To identify an epitope of the maximum possible activity the aa residues flanking

the consensus sequence found by phage display should be identified and, instead of cloning and expressing epitope mutants, 4-5 peptides of overlapping sequences with three aa shift of 15-20 aa in length should be synthesized and tested for specificity in a peptide ELISA. Further a set of 3-4 aa residues might be deleted (compound mutagenesis) simultaneously to identify residues required for optimal peptide activity. These synthesized peptides should provide the most optimal activity for the respective mAb as observed in this study.

The antigen candidates for unambiguous serological differential diagnostics should be rich in specificity determining residues. But at the same time aa residues of an epitope that may not contribute to the epitope binding directly, may be involved in contact, affinity, or affinity of discrimination[116]. Hence, to avoid mischaracterizing the residues, exhibiting such roles, that are often found adjacent to the critical aa residues, as non-critical, we for the first time, used a compound mutagenesis scanning approach in combination with phages display and peptide array epitope mapping, where a compound- additive or cooperative i.e. effect of multiple adjacent aa residues on specificity instead of studying the contribution of individual aa residues was studied. This approach may be applied to any epitope, of any protein of a known sequence, identified by a monoclonal antibody or polyclonal antibodies.

Limitations

Since the modifications of aa residues of an epitope is done in groups, it is not possible to determine the contribution of individual residues to the specificity or affinity of mAb binding. Our approach does not provide a comprehensive view of individual aa residue contribution to binding like site-directed mutagenesis, combinatorial or quantitative saturation mutagenesis[101-104]. Our approach is directed towards developing a comparatively less labor-intensive strategy that can provide with a domain level functional mapping of the epitope. In case an epitope

exclusively consists of multiple zones of weak interactions this approach may provide only limited information about the contribution of the individual domains of the epitopes. Our approach requires a prior knowledge of the complete sequence of the target protein and may have limited utility when working with mimotopes and conformational epitopes involving tertiary and/or quaternary structure of the antigen. However, tools like MIMOP, SAROTUP and MimoDB may be useful to trace epitopes from mimotope sequences[117-119].

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