

Exploring peptide mimics for the production of antibodies against discontinuous protein epitopes

Melita B. Irving^{a,1}, Lisa Craig^a, Alfredo Menendez^{a,2}, Beechanahalli P. Gangadhar^{a,3},
Marinieve Montero^{a,4}, Nienke E. van Houten^{b,**}, Jamie K. Scott^{a,b,*}

^a Department of Molecular Biology and Biochemistry, Simon Fraser University, 8888 University Dr., Burnaby, BC V5A 1S6, Canada

^b Faculty of Health Sciences, Simon Fraser University, 8888 University Dr., Burnaby, BC V5A 1S6, Canada

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ABSTRACT

Peptide “mimics” (mimotopes) of linear protein epitopes and carbohydrate epitopes have been successfully used as immunogens to elicit cross-reactive antibodies against their cognate epitopes; however, immunogenic mimicry has been difficult to achieve for discontinuous protein epitopes. To explore this, we developed from phage-displayed peptide libraries optimized peptide mimics for three well-characterized discontinuous epitopes on hen egg lysozyme and horse cytochrome c. The peptides competed with their cognate antigens for antibody binding, displayed affinities in the nM range, and shared critical binding residues with their native epitopes. Yet, while immunogenic, none of the peptides elicited antibodies that cross-reacted with their cognate antigens. We analyzed the 3-D structure of the site within each discontinuous epitope that shared critical binding residues with its peptide mimic, and observed that in each case it formed a ridge-like patch on the epitope; in no case did it cover most or all of the epitope. Thus, the peptides’ lack of immunogenic mimicry could be attributed to their inability to recapitulate the topological features of their cognate epitopes. Our results suggest that direct peptide immunizations are not a practical strategy for generating targeted antibody responses against discontinuous epitopes.

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1. Introduction

Neutralizing (Nt) antibodies (Abs) play an important role in protection against many infections and are elicited by most human vaccines (Zinkernagel, 2003). Yet, pathogens can evade Nt humoral immunity through the presentation of immunodominant, hyper-variable or repeated epitopes that play little or no role in protection (Nara and Garrity, 1998). Furthermore, Nt sites may be camouflaged by heavy glycosylation, or be transiently exposed during the infection process, as is the case of HIV-1 (Robinson, 2002).

While the immune system generates Abs in response to HIV-1 infection, they tend to be weakly Nt and strain-specific, and are easily evaded by minor antigenic variation of the viral envelope (Hermiston et al., 2003; Richman et al., 2003; Weiss et al., 1985). Consequently, only a few anti-HIV-1 Nt Abs have been identified that protect against a broad range of viral isolates (Hermiston et al., 2003; Parren et al., 1999; Walker et al., 2009). In addition, some Abs may have the undesired effect of enhancing microbial infection or pathogenesis (Blanco and Dirita, 2006; Fust, 1997; Subbramanian et al., 2002; Verrier et al., 2000), thus presenting a serious challenge in the development of Nt Ab-based vaccines. A means to develop immunogens that specifically target the production of effective Abs against neutralization-sensitive sites on pathogens would mark a critical advance in vaccine design.

It has been proposed that peptides representing or mimicking neutralization-sensitive sites on a pathogen might provide a way of focusing the production of Nt Abs against epitopes that are not immunodominant in the context of the whole antigen (Ag), hence the concept of peptide-based epitope-targeting vaccines (Beale, 1982; Green et al., 1982). Several strategies have been used to identify peptide mimics of Ab epitopes: (i) “rational design” (Eichler, 2004; Villen et al., 2002, 2006), (ii) selection from a panel of synthetic peptides derived from the linear sequence of the protein

* Corresponding author at: Department of Molecular Biology and Biochemistry, Simon Fraser University, 8888 University Dr., Burnaby, BC V5A 1S6, Canada. Tel.: +1 778 782 5658; fax: +1 778 782 5583.

** Corresponding author.

E-mail addresses: nvanhout@sfu.ca (N.E. van Houten), jkscott@sfu.ca (J.K. Scott).

¹ Current address: Multidisciplinary Oncology Center (CePO), Division of Experimental Oncology, 155 Chemin des Boveresses, Epalinges 1066, Switzerland.

² Current address: Michael Smith Laboratories, The University of British Columbia, #301-2185 East Mall, Vancouver, BC V6T 1Z4, Canada.

³ Current address: Polypeptide Laboratories Inc., 365 Maple Ave, Torrance, CA 90503, USA.

⁴ Current address: Child & Family Research Institute, University of British Columbia, Division of Gastroenterology, Department of Pediatrics, Rm 201, 950 West 28th Ave., Vancouver, BC V5Z 4H4, Canada.

(Getzoff et al., 1987), (iii) selection from a gene-fragment library derived from a gene encoding the target Ag (Fack et al., 1997; Matthews et al., 2002), or (iv) selection from phage-displayed peptide libraries (PDPLs) (Irving et al., 2001; Scott and Craig, 1994; van Houten and Scott, 2005). Ab should react with both the peptide and its cognate epitope, and cross-reactive peptides developed by these means should elicit Abs that bind the targeted epitope, neutralize the pathogen *in vitro*, and ultimately provide protection *in vivo*.

While simple in concept, epitope-targeted vaccines have proven difficult to develop, especially for discontinuous protein epitopes; this is of concern as the majority of protein epitopes are discontinuous (Jin et al., 1992; Van Regenmortel, 1996). They are differentiated from linear epitopes in comprising separate strands bearing critical binding residues (CBRs; i.e., residues required for Ab binding) that are brought together by protein folding (Barlow et al., 1986), and are best defined from crystallographic structures of Ab–Ag complexes or amino acid replacement studies (Jin et al., 1992). Discontinuous epitopes are dependent upon protein folding, and cannot be mapped with peptides representing the linear sequence of the Ag, however, this is also true of some linear epitopes.

Immunogenic mimics of linear epitopes have been derived directly from the linear sequences of proteins (Arnon and Van Regenmortel, 1992; Bastien et al., 1997; Green et al., 1982; Matthews et al., 2002; Moynihan et al., 2001; Niman et al., 1983; Watanabe et al., 1999; Wilson et al., 1984), as well as from PDPLs by selection with monoclonal (M)Abs (Arnon et al., 2000; Demangel et al., 1996; Grabowska et al., 2000; Scala et al., 1999; Schellekens et al., 1994; Yu et al., 2000). Most instances in which peptides selected from PDPLs conferred protection *in vivo* against challenge with the corresponding pathogen (Arnon et al., 2000; Grabowska et al., 2000; Schellekens et al., 1994; Steward et al., 1995; Yu et al., 2000) involve linear epitopes.

To date there have been few reports of peptides, either selected from PDPLs (Dorgham et al., 2005; Matthews et al., 2002) or rationally designed (Villen et al., 2006), that act as immunogenic mimics of discontinuous protein epitopes. The reasons for this are not fully understood, but may relate to an inability of peptides to faithfully replicate the structural features of a native Ag (Irving et al., 2001; van Houten and Scott, 2005), and/or to the fact that the Ag-binding sites of anti-protein and anti-peptide Abs are structurally different (MacCallum et al., 1996). In the work presented here, we studied the ability of peptide mimics of three well-characterized discontinuous protein epitopes (Fischmann et al., 1991; Mylvaganam et al., 1998; Sheriff et al., 1987) to act as immunogenic mimics. Two MABs against hen egg lysozyme (HEL; D1.3; Bhat et al., 1990; Harper et al., 1987) and HyHEL-5 (Smith-Gill et al., 1984, 1982), and a MAB against horse cytochrome c (CytC; E8; Carbone and Paterson, 1985), were used to isolate and optimize peptides from PDPLs. For each MAB, peptides were identified that competed with cognate Ag for Ab binding, and mice immunized with phage displaying the peptides produced significant anti-peptide Ab responses. However, none of the immune sera cross-reacted with the cognate Ags (HEL or CytC), none of the peptides were immunogenic mimics. These failures were not due to low peptide affinity, or the lack of shared CBRs between each peptide and its cognate epitope. However, structural analysis of the shared CBRs in the context of each cognate epitope revealed that in every case the CBRs formed a small ridge-like patch within each larger epitope, potentially explaining another aspect of each peptide's cross-reactivity, and its inability to elicit cross-reactive Abs. We conclude that, while candidate peptides isolated from PDPLs can be high-affinity, Ab-specific ligands, their use in targeting the Ab response against discontinuous epitopes is unlikely to be effective because they do not mimic the full cognate epitope.

2. Materials and methods

2.1. Materials

D1.3 Fab and IgG were generous gifts from Dr. R. Mariuzza (University of Maryland Biotechnology Institute, Rockville, MD). Additional D1.3 IgG was kindly provided by Dr. R. Willson (University of Houston, TX). E8 IgG was a gift from Dr. E. Getzoff (The Scripps Research Institute, La Jolla, CA). Hy-HEL-5 IgG was a gift from Dr. S. J. Smith-Gill (National Cancer Institute, Frederick, MD). NHS-LC-biotin was from Pierce Biotechnology Inc. (Rockford, IL). *Escherichia coli* strain MC1061 was used in the production of phage libraries and for site-directed mutagenesis. Oligonucleotides were synthesized at the NAPS Unit (University of British Columbia, Vancouver) or “in-house” using an ABI 392 Oligo Synthesizer (Applied Biosystems, Foster City, CA). Unless otherwise indicated, all reagents required for DNA manipulations were obtained from New England BioLabs (NEB; Ipswich, MA). HEL, bovine serum albumin (BSA) and ovalbumin (OVA) were purchased from Sigma–Aldrich (St. Louis, MO). Synthetic peptide, D1.3p-E, was purchased from Multiple Peptide Systems (San Diego, CA) (see Supplemental Table 1 for the sequences of all synthetic peptides). Synthetic peptides, D1.3p-C, E8p and all of the E8p peptides bearing amino acid replacements were synthesized “in-house” using a multi-pin system from Chiron Mimotopes PTY Ltd. (Victoria, Australia) and Fmoc chemistry following the manufacturer's instruction. The peptides were cyclized, amidated at their carboxy termini, and HPLC-purified to >95% purity. Streptavidin (SA) was purchased from Roche Diagnostics (Indianapolis, IN). The adjuvants, Adjuvax (Alpha-Beta Technology, Worcester, MA), Imject® Alum (Pierce) and MPL® + TDM (Sigma–Aldrich) were used as directed.

2.2. Mice and immunizations

Female BALB/c mice aged 6–8 weeks were purchased from Charles River Laboratories (St. Constant, QC) and allowed to mature at the Animal Care Facility at Simon Fraser University. All animal studies were conducted in accordance with the Canadian Council on Animal Care and institutional guidelines.

2.2.1. Protein immunizations

One group of mice ($n=10$) was immunized every second week *via* intraperitoneal (i.p.) injection with a 100 μ l volume of phosphate-buffered saline (PBS) containing Adjuvax adjuvant and 50 μ g HEL. Two groups of mice ($n=10$) received subcutaneous (s.c.) injections every second week with a total volume of 100 μ l PBS containing 25 μ g HEL and either Imject® Alum or MPL® + TDM adjuvant. A control group ($n=5$) was immunized *via* s.c. injection every second week with 50 μ l PBS containing 25 μ g OVA and an equal volume of Imject® Alum. Mice were bled from the tail prior to each immunization. Final bleeds were performed by cardiac puncture under CO₂ anaesthesia.

2.2.2. Phage immunizations

Prior to immunization, phage clones bearing recombinant peptides were purified by CsCl equilibrium density gradient centrifugation (Bonnycastle et al., 2001). For immunizations with phage, groups of mice ($n=4$) received 100 μ l PBS containing 3×10^{11} phage particles (corresponding to 10 μ g amount of protein) *via* s.c. injection every second week. Four immunizations were conducted and no adjuvant was used. Mice were bled from the tail or the saphenous vein prior to each immunization. Seven to 10 days after the fourth immunization, the mice were bled by cardiac puncture under CO₂ anaesthesia.

2.3. Probing of overlapping 10-mer HEL peptides with anti-HEL sera

A set of 10-mer HEL peptides, overlapping every second residue and covering the entire sequence of HEL, was synthesized on a cellulose membrane by PepMetric Technologies Inc. (Vancouver, BC). The peptides were prepared in duplicate on the same membrane; the only exception to the HEL sequence is that Cys residues were replaced with Ser. The peptides were analyzed with five anti-HEL serum samples as well as with a non-immune serum, anti-OVA serum, and secondary Ab (1 in 2500 dilution) by itself. To probe the peptides, the membrane was first hydrated by treating it with methanol, then 50% (v/v) methanol in water followed by three washes in 50 mM Tris containing 0.2% Tw. The membrane was subsequently blocked overnight (ON) in PBS containing 5% (w/v) non-fat dried milk (NFDM) and then washed once in PBS containing 0.2% Tw (PBS/Tw) for 15 min prior to incubation with 1:100 dilution mouse serum in PBS/Tw/NFDM, for 2 h at RT. The membrane was then washed for 5 min three times in PBS/Tw prior to the incubation with HRP-conjugated-goat-(anti-mouse-IgG-Fc)-Ab diluted 1:2500 in PBS/Tw/NFDM, for 2 h at RT. The membrane was thoroughly washed in PBS/Tw and developed with the Western Lightning Chemiluminescence Kit (PerkinElmer, Wellesley, MA) according to the manufacturer's instructions, and exposed to fast Fuji X-ray film. The membrane was regenerated by incubation ON in 8 M urea containing 1% SDS, followed by a 1-h incubation in 50% ethanol/10% acetic acid, and three washes in methanol.

2.4. PDPLs, their screening and phage DNA sequencing

A panel of 14 primary PDPLs (constructed in phage vector, f88-4; Zhong et al., 1994) displaying randomized peptides at the N-terminus of pVIII was screened with MAb D1.3, HyHEL-5 and E8. The PDPLs comprise random peptide sequences ranging in length from 6 to 15 amino acids, with some bearing one or two fixed Cys residues (Bonnycastle et al., 1996). The sequences of the randomized peptides in each library are: X₆, X₁₅, X₈CX₈, X₁₅CX, XCX₁₅, XCX₈CXGG, XCX₁₀CXGG, XCX₁₂CXGG, X₅CX₃CX₅, XCX₄CX, X₄CX₄CX₄, X₄CX₅CX₄, X₄CX₆CX₄ and XCX₆CX (where X is a randomized residue and C is a fixed Cys), and are followed by one of three fixed amino acid sequences, PAEG, GGPAEG or AAEG, at the C-terminus (AEG forms the N-terminus of wild type (wt) pVIII). The latter five libraries were a gift from G.P. Smith (University of Missouri, Columbia).

As summarized in Supplemental Table 2, each primary PDPL or sublibrary was screened using biotinylated (bio-) IgG or bio-Fab in three or four rounds of solid-phase and/or in-solution biopanning, as described in Menendez et al. (2001). The PDPLs were first “pre-adsorbed” on SA-magnetic beads or in SA-coated microtiter plate wells (Bonnycastle et al., 1996; Menendez and Scott, 2005). For in-solution selections, bio-Abs were incubated with phage particles and then bio-Ab-phage complexes were captured on SA-coated magnetic beads (Dynal, Lake Success, NY). For solid-phase selections, bio-Ab was immobilized on SA-coated microtiter plate wells or on SA-coated beads, then 10¹¹ phage particles were incubated for 2 h with bio-MAb. All incubations were performed in Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl). The SA-coated beads or microplate wells bearing captured bio-Ab-phage complexes were washed 3 times in TBS buffer containing 0.1% (v/v) Tween 20 (TBS/Tw), then phage particles were eluted with 50 μ l elution buffer (0.1 M glycine-HCl, 1% (w/v) BSA, pH 2.2) immediately followed by neutralization with 10 μ l 1 M Tris-HCl, pH 9.1.

After each round of screening, eluted phage pools were amplified by infection of starved *E. coli* strain K91 cells and the phage yields calculated (Menendez et al., 2001). Individual colonies producing phage clones were isolated on NZY agar containing 40 μ g/ml

tetracycline (Tet), and amplified in 2-ml cultures of NZY containing 20 μ g/ml Tet and 1 mM IPTG. Phage were precipitated from culture supernatants with 0.15 volume PEG/NaCl, resuspended in TBS, and phage concentrations were estimated by gel electrophoresis (0.8% (w/v) agarose, 4 $\times$ GBB buffer) and by UV spectroscopy at A₂₆₉. Phage particles were purified by precipitation in NaCl and polyethylene glycol (PEG), resuspended in TBS and tested for Ab binding in direct ELISA; viral DNA encoding their recombinant peptides was sequenced. All general phage methods are described in detail in Bonnycastle et al. (2001). Phage sublibraries were constructed using the vector, f88-4, as described (Bonnycastle et al., 1996), and were screened with bio-IgG and/or bio-Fab (see Supplemental Table 2).

DNA sequencing reactions were performed with the Thermo Sequenase II Dye Terminator Cycle Kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's instructions. DNA fragments were resolved on an ABI Prism 373 DNA sequencer (Applied Biosystems) and analyzed with EditView 1.0.1 software.

2.5. Direct, competition and serum titration ELISAs

Direct phage ELISAs were conducted as described (Zwick et al., 2001a,b). Briefly, microtiter plate wells (Costar polystyrene, high-binding, Corning, NY) were coated with 0.2 μ g peptide suspended in 35 μ l TBS per well, and incubated in an uncovered plate for 4–6 h at 37 °C, or until the wells had dried completely. Phage particles (2 $\times$ 10¹⁰ per well) and proteins, including HEL, OVA and SA (1 μ g per well), were coated ON at 4 °C in 35 μ l TBS per well. Wells were then blocked for 30 min at 37 °C with 200 μ l TBS containing 2% BSA. After three washes with TBS/Tw, 35 μ l primary Ab diluted in TBS/Tw containing 1% BSA was added to each well and incubated for 2 h at RT. The plates were washed six times with TBS/Tw, followed by the addition of secondary reagent diluted in TBS/Tw containing 1% BSA. For the detection of murine MAb and sera, HRP-conjugated goat-(anti-mouse-IgG)-Fc or HRP-conjugated goat-(anti-mouse IgG-H + L)-Ab (Pierce, Rockford, IL), diluted 1:2000, was added to wells and incubated for 45 min at RT; bio-Ab was detected with HRP-conjugated-neutravidin, diluted 1:1500 (NA, Pierce). Bound HRP was detected with 0.03% (v/v) H₂O₂ and 400 μ g/ml 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in citrate/phosphate buffer (Bonnycastle et al., 1996), and the absorbance was measured (typically at 15, 30 and 45 min) on a VersaMax plate reader (Molecular Devices, Sunnyvale, CA) at 405 and 490 nm. Unless otherwise stated, the data are reported as O.D._{405–490} after 30 min.

For competition ELISAs, the direct ELISA procedure was followed except that the primary MAb or sera was incubated with: (i) 5–10 μ M HEL, OVA or CytC; (ii) up to 1 mM peptide; or (iii) 2 $\times$ 10¹⁰ phage particles, for a minimum of 2 h at RT, or overnight at 4 °C. Serum titration ELISAs were performed as described by van Houten et al. (2006).

For competition ELISAs with the E8p peptide and those bearing amino acid replacements (Supplemental Table 1), microwells were coated overnight at 4 °C with 1 $\times$ 10¹⁰ E8 phage particles or 2 μ g CytC in 35 μ l TBS, then blocked with TBS buffer containing 2% BSA. The following day, 35 μ l containing 200 μ M peptide and 3 nM E8 Fab were incubated for 4 h at RT, then added to BSA-blocked microwells. After 2 h the plates were washed and bound Fab detected with HRP-conjugated goat-(anti-mouse IgG-H + L)-Ab and developed as described.

2.6. Production D1.3-E-MBP fusion protein

The pMal-X vector is a modified version of the maltose binding protein (MBP) expression vector, pMal-p2 (New England Biolabs; Beverly, MA) (Zwick et al., 1998); it is designed to fuse recombinant

peptides and proteins to the N-terminus of MBP. DNA encoding the D1.3p-E peptide was transferred to the pMal-X vector and the D1.3-E-MBP fusion protein was produced in *E. coli* strain ER2507 and purified on amylose columns as described in Zwick et al. (1998). Protein concentrations were determined using the Protein Assay Kit from BioRad (Hercules, CA).

2.7. Determination of peptide affinity by kinetic exclusion assay

The KinExA instrument (kinetic exclusion assay, Sapidyn Instruments Inc., Boise, ID) was used to determine K_D s; it measures the concentration of free Ab in Ag–Ab reactions at equilibrium (Blake et al., 1999). The amount of free Ab is determined by flowing Ag–Ab equilibrium reactions over Ag immobilized on polymethylmethacrylate beads. The amount of free Ab that binds to the solid beads, as measured by fluorescence emission from a fluorescently labeled secondary Ab, is directly proportional to the concentration of free Ab in the equilibrium phase. The KinExA immunoassay was performed as described in Craig (1998) except that the beads were coated with bio-HEL. To set up the Ag–Ab equilibrium reactions, serial dilutions of synthetic D1.3p-E or D1.3p-C peptide, HEL or D1.3p-E-MBP fusion protein were incubated with 10–20 nM D1.3 Fab or IgG and incubated ON at 4 °C. Reactions were then flowed over the beads, which were then washed, and bound Ab was measured after incubation with a 1:1000 dilution of Cy5-conjugated-goat-(anti-mouse-IgG-H+L) IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Fluorescence data were analyzed with software provided by the manufacturer and plotted as a function of Ag concentration. The best-fit values for the K_D , and the concentration of Ab active sites were calculated, as well as the 95% confidence interval for these values. All experiments were conducted in duplicate and the mean values are reported.

2.8. Site-directed mutagenesis of D1.3-E phage clone for Ala replacement study

Site-directed mutagenesis reactions for generating Ala substitutions in phage-displayed peptides used as template covalently closed, circular, single-stranded phage DNA isolated from the D1.3-E phage clone; mutagenesis reactions were performed as described in Sambrook et al. (1989).

3. Results

3.1. Peptide ligands for MAb D1.3

The MAb D1.3-HEL complex is among the best-characterized Ab–Ag interactions. To identify peptide ligands for MAb D1.3, a panel of 14 primary PDPLs was screened with D1.3 (see Supplemental Table 2 for screening conditions). After several rounds of screening, individual phage clones were selected from pools that were positive for Ab-binding and tested in ELISAs for their ability bind D1.3; their displayed peptide sequences were deduced from the DNA sequencing of recombinant gene 8. Multiple clones were selected, which grouped into two unrelated consensus sequences, $X_3NCXGX_2CXRX_2$ and $XCXPQ(T/S/A)(T/S/A)X_5CX$. Two sublibraries (E and C) were constructed following the consensus sequences and screened under more stringent conditions to select tighter binding clones, yielding additional consensus residues (Table 1A, for consensus plots see Supplemental Figure 1). The strongest binding peptides from each sublibrary (E3R2.4 from sublibrary E and C4R3.14 from sublibrary C) were chosen for further study, renamed D1.3p-E and D1.3p-C, respectively, and prepared as synthetic peptides. Both peptides bound at or near the D1.3

Table 1

Peptide ligands from PDPL screenings with D1.3 MAb: Sequences and ELISA data from phage clones (A), competition ELISA with synthetic peptides (B), affinities as measured by KinExA (C).

A				
Phage Clone	Amino Acid Sequence	O.D. _{405–490} ^a		
Sublibrary E ^b : X ₃ NCXGX ₂ CXRX ₂				
E3R2.4 (D1.3p-E) ^c	SQPNCDGLVCNRWH ^d	1.14		
E3R2.5	GSSNCDGGSCVRRH	1.10		
E3R2.15	PVSNCDSGNCIRYH	1.06		
E3R2.10	RVRNCDGKYCVRS ^h	1.04		
E3R3.8	DLKNCDGEACVRWH	1.04		
E3R2.2	GSSNCDGGSCVRRH	0.94		
E3R2.7	LTTNCDGQACIRWH	0.92		
E3R2.16	PLPNCDGASCVRRS	0.76		
E3R2.18	YPPNCDGSYCFRIH	0.63		
55E.3	SNRNCDGHYCLRMG ^e	0.57		
Sublibrary C ^b : XCXPQ(T/S/A)(T/S/A)X ₅ CX				
C4R3.14 (D1.3p-C) ^f	TCYPQTSITPPHCD ^d	0.35		
C4R3.8	RCYPQSSITPPYCD	0.27		
55C.4	QCRPQTSKLPAMCS ^e	0.10		
f88.4	–	0.01		
B				
Immobilized Antigen	D1.3 Fab ^g	D1.3 Fab + D1.3p-E peptide	D1.3 Fab + D1.3p-C peptide	
D1.3p-E peptide	0.50	0.03	0.02	
D1.3p-C peptide	0.17	0.02	0.02	
HEL	0.86	0.01	0.01	
C				
Ag-Ab Interaction	K _D Value	% Error ^h	K _D high	K _D low
HEL and D1.3 Fab	3.4 nM	2.8	4.4 nM	0.14 nM
D1.3p-C synthetic peptide and D1.3 Fab	6.2 μM	1.6	7.5 μM	4.7 μM
D1.3p-C synthetic peptide and D1.3 IgG	4.1 μM	4.1	8 μM	1.5 μM
D1.3p-E synthetic peptide and D1.3 Fab	1.7 μM	4.4	2.4 μM	1.9 μM
D1.3p-E-MBP fusion protein and D1.3 Fab	31 nM	4.8	44 nM	13 nM

^a Primary Ab: 100 nM D1.3 Fab; secondary reagent: HRP-NA. The data are from one of three representative experiments.

^b Amino-acid sequence of sublibraries E and C. In bold are consensus residues that were identified from the primary PDPL screenings and fixed in the sublibraries.

^c Synthetic peptide D1.3p-E was based on this sequence.

^d Underlined are consensus residues identified from screening sublibraries E and C.

^e Sequences of the parental clones from which sublibraries E and C were derived.

^f Synthetic peptide D1.3p-C was based on this sequence.

^g Primary Ab: 20 nM D1.3; secondary reagent: HRP-conjugated goat-(anti-mouse-IgG-H+L)-IgG. For competitions, 1 mM D1.3p-E peptide or D1.3p-C peptide was reacted with D1.3 Fab ON at 4 °C before incubation with immobilized antigen.

^h The % error based on the root-mean square deviation of the data from the theoretical best-fit curve. K_D high and K_D low are the boundaries of the 95% confidence interval for the K_D estimate. All values are averaged from two experiments.

paratope, as competition ELISA showed that each competed with HEL and with each other for binding to D1.3 Fab (Table 1B).

The recombinant phage and peptide ligands D1.3p-E and D1.3p-C were used to probe the polyclonal Ab response against HEL for “D1.3-like” specificities. BALB/c mice were immunized with HEL, eliciting a strong Ab response (Supplemental Figure 2). However, anti-HEL sera did not bind to recombinant phage nor to synthetic peptide ligands of D1.3, indicating that D1.3-like specificities are rare in the polyclonal Ab response against HEL (data not shown). However, reactivity of five different anti-HEL sera with a set of overlapping, 10-mer HEL peptides immobilized to a membrane (covering the whole HEL sequence) showed that the Ab specificities against HEL were numerous and highly conserved amongst

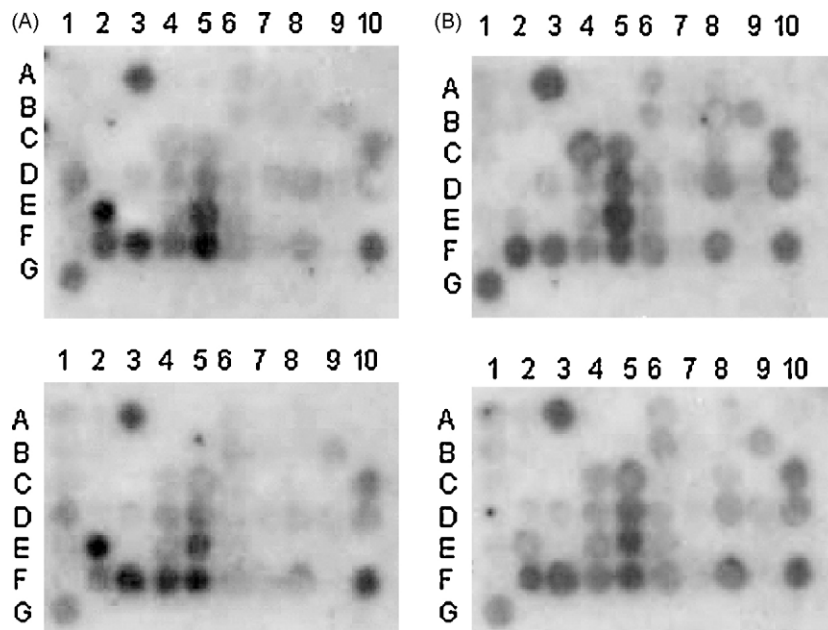


Fig. 1. Overlapping 10-mer peptides covering the HEL amino acid sequence probed with anti-HEL sera reveals common patterns of reactivity. Shown are images from X-ray film exposed to membrane incubated with two different anti-HEL serum samples (A and B). The top and bottom images are duplicates of each set of HEL overlapping peptides. For corresponding peptide sequences see [Supplemental Table 3](#).

mice ([Fig. 1](#), see [Supplemental Table 3](#) for peptide sequences and relative binding by sera). Collectively, these results validate the D1.3-HEL system as an exquisite model of a real vaccine situation since the D1.3-binding site on HEL is a poorly immunogenic epitope as compared to numerous other immunodominant sites, much like many neutralization-sensitive sites of pathogens such as HIV-1. Thus D1.3-HEL constitutes a good system to address the feasibility of using peptide ligands to elicit targeted Ab responses.

3.2. Peptide ligands for MAbs D1.3 do not elicit Abs that cross-react with HEL

The ability D1.3p-E and D1.3p-C peptides to act as immunogenic mimics of the D1.3 epitope was tested by immunizing BALB/c mice with the D1.3p-E and D1.3p-C recombinant phage. Sera were analyzed for binding to phage, synthetic peptide and non-specific Ags in titration ELISA after the second (data not shown) and fourth immunizations ([Fig. 2](#); serum titration curves are shown in [Supplemental Figure 3A and B](#)). Anti-phage Ab responses with average half-maximum titers ranging from 10,000 to 125,000 were generated by all immunized mice. In addition, anti-peptide Ab titers ranging from 400 to 1800 were generated by mice immunized with phage clones D1.3p-E and D1.3p-C. However, in spite of the presence of anti-peptide Ab responses, none of the immunized mice produced Abs that cross-reacted with HEL. Moreover, the D1.3p-E phage immunizations did not yield sera that cross-reacted with D1.3p-C peptide, nor did anti-D1.3p-C sera cross-react with the D1.3p-E peptide ([Supplemental Figure 3A and B](#)). Thus, although both peptides were immunogenic, neither one acted as an immunogenic mimic of the D1.3 epitope on HEL.

3.3. Affinity of D1.3 for its peptide ligands

It is commonly thought that affinity correlates with immunogenicity of an Ag ([Agarwal et al., 1998](#); [Agarwal and Rao, 1997](#); [Nakra et al., 2000](#)). As none of the peptides elicited a serum

response that cross-reacted with HEL, we sought to determine the affinity of D1.3 binding to the D1.3p-E and D1.3p-C peptides relative to the native Ag, HEL. Several K_D values have been published for the D1.3-HEL complex; [England et al. \(1999\)](#), reported a K_D of 11.5 ± 1.9 nM and [Hawkins et al. \(1993\)](#), reported a K_D of 3.3 nM. [Table 1C](#) shows the K_D values and ranges obtained for D1.3 binding to HEL and the synthetic peptides D1.3p-E and the D1.3p-C as measured with the KinExA instrument. As an example of a typical experiment, kinetic data for synthetic D1.3p-C are shown in [Supplemental Figure 4](#). D1.3 (Fab)-HEL affinity was 3.4 nM, well within the range previously reported ([Hawkins et al., 1993](#)). The interaction with synthetic peptides D1.3p-E and D1.3p-C was much weaker, with K_D values of 1.7 and 6.2 μ M, respectively.

Previous work by our group and others has shown that a significant drop in Ab-binding affinity is observed when recombinantly displayed peptides are replaced by their free synthetic

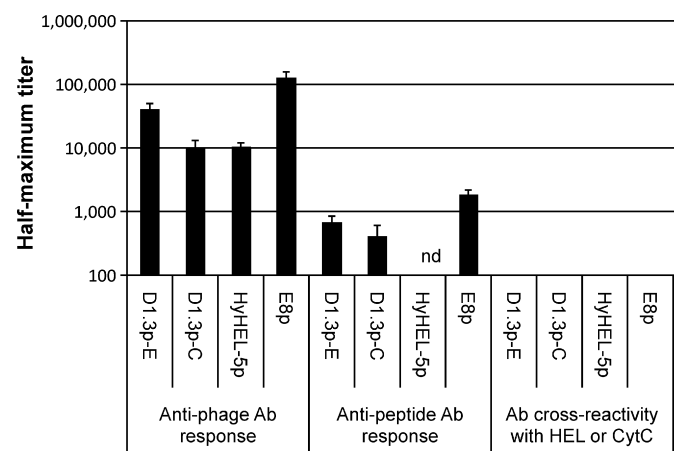


Fig. 2. Average half-maximum Ab titers after four immunizations against phage, synthetic peptide, and cognate antigen produced by mice immunized with phage clones D1.3p-E, D1.3p-C, HyHEL-5p and E8p. nd: Not done, this synthetic peptide could not be made.

Table 2

Sequences and competition ELISA of phage clones selected with HyHEL-5 MAb from the primary PDPLs and from sublibrary A.

Phage clone	Peptide sequence	HyHEL-5 ^a	HyHEL-5 + HEL ^b
Sublibrary A^c: XCVGXRGX₆RCX			
A9.2 (HyHEL-5p) ^d	T CVGS RG DFMPHPRC G	1.00	0.01
A7.4	SCRRPSDSHSCD	0.69	0.01
A7.1	A CVGH RG Pt R CL	0.56	0.01
f88.4	–	0.01	nd ^e

^a Direct ELISA with 100 nM HyHEL-5 IgG. HRP-NA conjugate was the secondary reagent. The data are from one of two representative experiments.

^b For competitions, HyHEL-5 IgG was reacted with 5 μM HEL for 2 h at RT before incubation with immobilized phage.

^c Amino acid sequence of sublibrary A.

^d Clone HyHEL-5p was isolated from sublibrary A; clones A7.4 and A7.1 were isolated from the primary PDPLs.

^e nd, Not done.

counterparts (Chirinos-Rojas et al., 1999; Delmastro et al., 1997; Menendez et al., 2008; Saphire et al., 2007; Zwick et al., 2001a,b). Thus, synthetic peptide affinities do not necessarily reflect the affinity for peptides in the context of the phage (which were used as immunogens). The peptide that bound best to D1.3 MAb, D1.3p-E, was fused to the amino terminus of MBP (D1.3p-E-MBP) for affinity analysis, in order to gauge the differences in D1.3 MAb affinity for synthetic versus recombinant, N-terminal peptide. The K_D of D1.3 Fab binding to D1.3p-E-MBP fusion protein was determined to be 31 nM, only 10-fold weaker than the affinity of D1.3 Fab for HEL (Table 1C). This agrees with previous results in other Ab–Ag and Ab–peptide systems, and suggests that the phage-borne versions of the D1.3 peptide ligands display an affinity for D1.3 close to that of HEL. The D1.3p-E peptide was further optimized by screening a sublibrary based on the nucleotide sequence encoding D1.3p-E “doped” with NNK (Supplemental Table 4) degenerate oligonucleotides, and some clones from it appeared to bind D1.3 Fab more strongly than HEL itself. Thus, this peptide, fully optimized, probably has an affinity near that of HEL.

3.4. Peptide ligands for MAb HyHEL5 and E8 do not elicit HEL or CytC cross-reacting Abs, respectively

To extend this study beyond the D1.3–HEL model, a second HEL-specific MAb, HyHEL5 (Cohen et al., 1996; Sheriff et al., 1987) was used to screen the primary PDPLs (Supplemental Table 2), HyHEL5 binds to a well-characterized epitope (Smith-Gill et al., 1984, 1982) that does not overlap with that of the D1.3 MAb. As shown in Table 2, shared residues between two peptides (A9.2 and A7.1) were identified and used to produce a sublibrary having the sequence: XCVGXRGX₆RCX (consensus residues are in bold). Peptide ligands for HyHEL5 obtained from this library (Supplemental Table 5) did not produce additional consensus residues, and did not bind substantially better than the “parental” clone, A9.2, which was chosen for further study and renamed HyHEL5p. Immunizations of BALB/c mice with phage HyHEL5p induced strong phage-binding Abs, and presumably, peptide binding Abs, but no reactivity with HEL (Fig. 2 and Supplemental Figure 3C). The anti-peptide Ab response was not assessed, as repeated attempts to synthesize HyHEL5p were unsuccessful.

The third MAb analyzed, E8, is against a well-characterized discontinuous epitope on horse CytC. Screening of the primary PDPLs with MAb E8 selected binding clones with shared residues (data not shown), from which a sublibrary with the sequence, XCX₂DDX₄CX, was constructed and screened under stringent conditions. Multiple clones showed an expanded consensus sequence (XCPPDDXKXLCX) and significant binding to E8 IgG (Table 3A); several of those clones also showed par-

Table 3

A, Sequences and ELISA of phage clones selected with E8 IgG from the E8 sublibrary. XCX₂DDX₄CX B, Competition ELISA measuring the effect of phage clone E8p on the binding of E8 IgG to CytC.

A		
Phage Clone	Sequence	E8 IgG ^a
3B.9	RCPPDDTKRLCW	0.37
3A.2 (E8p)	MCPDDTKRLCI ^b	0.35
3C.15	ICPPDDTKNLCH	0.27
3A.9	TCPPDDTKGLCS	0.26
3A.5	ICPPDDDKGLCM	0.25
3B.19	MCPDDIKALCR	0.20
3C.8	VCPDDSKMLCA	0.17
3A.14	SCPPDDTKLLCT	0.13
3B.20	RCPPDDHKKLCM	0.13
3B.8	NCPPDDYKRLCV	0.10
3C.6	MCPDDSKLLCA	0.05
3A.1	ECPPDDTKYLCS	0.05
3A.3	HCPPDDIKHLCO	0.05
3A.7	NCPPDDYKHLCV	0.03
3B.4	ACPPDDTKSLCF	0.03
3C.3	RCPPDDTMALCR	0.02
3A.4	ACHKDDTLPICA	0.01
3A.8	RCPPDDTARHCR	0.01
3B.1	TCPPDDSRSLCR	0.01
3B.12	GCPPDDLKTLCI	0.01
f88-4	–	0.01
B		
Competitor ^c	Peptide Sequence	E8 + Competitor ^d
None	–	0.55 ^e
3B.9	RCPPDDTKRLCW	0.34
3A.2 (E8p)	MCPDDTKRLCI	0.39
3C.15	ICPPDDTKNLCH	0.40
3A.9	TCPPDDTKGLCS	0.38
3A.5	ICPPDDDKGLCM	0.37
f88.4	–	0.51
CytC	–	0.27

^a Primary Ab: 100 nM E8 Fab; secondary reagent: HRP-neutravidin.

^b Synthetic peptide E8p was based on this sequence. It was not possible to synthesize a peptide based on the sequence of the tightest-binding phage clone, 3B.9. Phage clone 3A.2 was used for immunization. Consensus residues are in bold.

^c Phage clones (3×10^{10} particles) or 5 μM CytC were reacted with 100 nM E8 IgG for 2 h, then incubated with wells coated with CytC.

^d Binding of bio-E8 IgG to plate-captured CytC was detected with SA-HRP conjugate.

^e 100% signal of bio-E8 IgG against CytC with no competitor.

tial competition with immobilized CytC for binding to E8 IgG (Table 3B). Clone 3A.2 was selected for further study and renamed E8p. Mice immunized with E8p recombinant phage (Figure 1, Supplemental Figure 3D) produced Abs that reacted with synthetic E8 peptide. However, no reactivity against CytC was observed.

3.5. CBRs on peptides D1.3p-E and E8p

A series of recombinant phage mutants was constructed in which each amino acid of the D1.3p-E peptide was individually replaced with Ala. Phage clones bearing single Ala substitutions were then tested in direct ELISA for binding to D1.3 Fab with respect to the parental D1.3p-E phage clone (Fig. 3A). Individual replacements of Asn4, Cys5, Asp6, Gly7, Cys10, Arg12, His14 and Pro15 significantly decreased binding to D1.3 Fab (>80%), defining these residues as CBRs. As expected, D1.3 Fab binding showed greater sensitivity to the substitutions than the IgG. In addition, the intra-chain disulfide bridge between Cys5 and Cys10 was shown to be required, as D1.3 binding was ablated in direct phage ELISAs performed under reducing conditions (data not shown). The CBRs for D1.3p-E were independently confirmed by screening the NNK-doped D1.3p-E sublibrary; all of the CBRs

identified in the Ala replacement study were conserved among D1.3-binding clones isolated from this library (Supplemental Table 4).

The CBRs for the E8p peptide were determined using synthetic peptides bearing an Ala- or Ser-replacement at each amino acid position (with the exception of position 8; listed in Tables 1A–1C). The peptides were used in a competition ELISA in which MAb E8 was incubated with peptide, CytC or E8 phage, and then tested for binding to immobilized E8 phage and CytC; replacement of CBRs in the peptide should ablate a peptide's competitive activity. As shown in Fig. 3B, soluble peptides bearing the amino acid replacements, Cys2Ser, Pro3Ala, Asp5Ala, Asp6Ala, Leu10Ala and Cys11Ser, as well as the negative control, did not affect E8 MAb binding to immobilized Ag, confirming their CBR status. The peptide bearing the Lys8Ala replacement could not be synthesized, thus the role of this residue was not determined; however, Lys8 appeared as a consensus residue in the library screenings (Table 3A) and thus is probably important for Ab binding. Besides Lys8, the other CBRs identified in the competition study were also conserved in the sub-library screening.

3.6. Abs select peptides containing CBRs in common with their native epitopes

Using the co-crystal structures of the Ab/antigen complexes, we analyzed the extent to which cross-reactive peptides possess amino acids in common with residues in their known native epitopes, and compared their respective involvement in MAb binding (Tables 4A and 4B). The HEL-D1.3 interaction has been extensively characterized. Dall'Acqua et al. (1998) and Hawkins et al. (1993) have collectively shown that Gly117, Thr118, Asp119, Val120, Gln121 and Arg125 of HEL play critical roles in its interaction with D1.3 MAb (see Supplemental Table 6). Furthermore, Dall'Acqua et al., found that Gln121 of HEL, whose side chain protrudes into a small cleft between the third hypervariable loops of V_H and V_L, was the most important residue for binding by D1.3 MAb. Both D1.3p-C (TCYPQISTTPPHCD, consensus residues in bold) and D1.3p-E (SQPNCDGLVCNRWH) share amino acids with critical contacts in the D1.3 epitope on HEL (Table 4A). D1.3p-C shares two, Gln5 and Asp14, whereas D1.3p-E shares as many as four CBRs, Asn4, Asp6, Gly7 and Arg12, with Asn substituting for Gln.

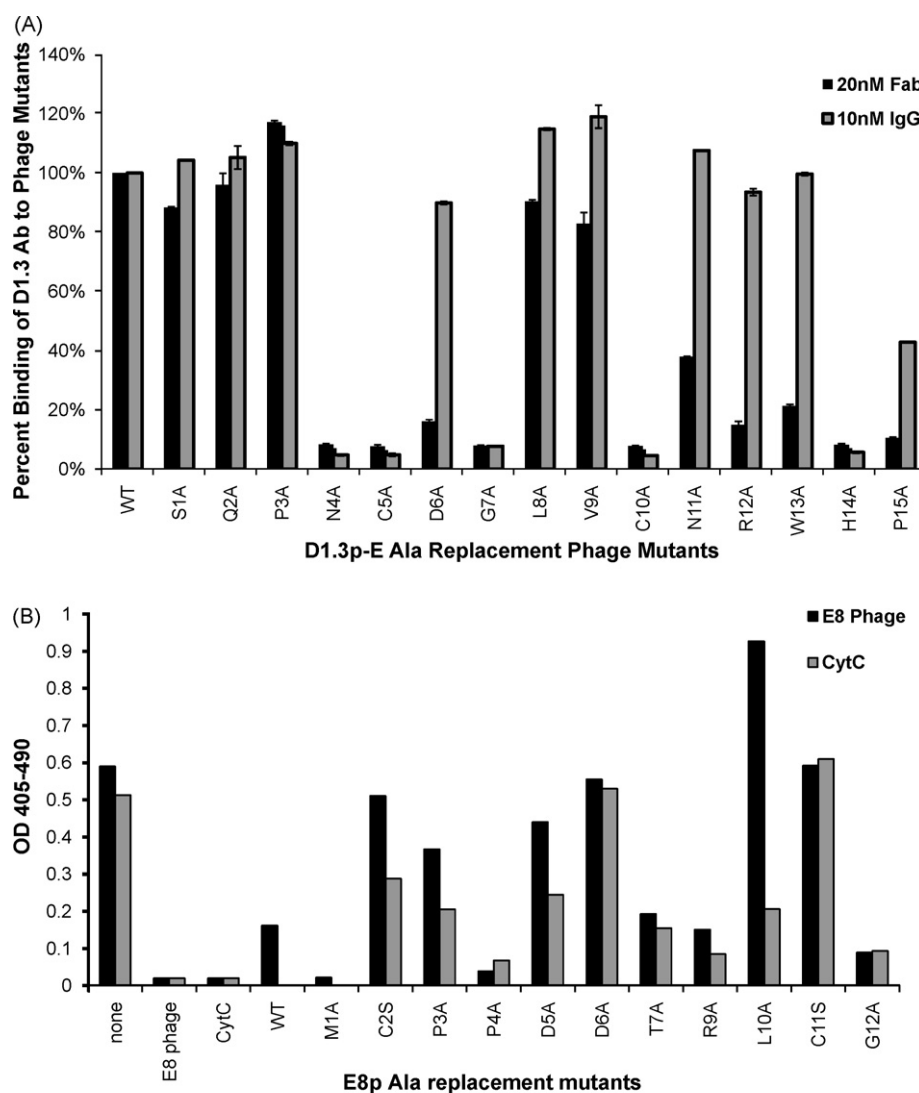


Fig. 3. Defining CBRs in peptides D1.3p-E and E8p. (A) Binding of D1.3 Fab and IgG with phage clones bearing Ala replacements in the D1.3-E phage peptide. (B) Competition for E8 Fab binding by E8 phage, CytC and synthetic E8 peptides bearing Ala or Ser replacements at each amino acid position versus immobilized E8 phage and CytC. A lack of competition by a peptides (i.e., high ELISA signal) shows that its replaced residue is critical to E8 Fab binding. ELISA readings were taken at 30 min, and HRP-conjugated-goat-(anti-mouse-IgG-H + L) Ab was used as the secondary reagent for both experiments.

Fig. 4A shows the surface topology of the epitope for D1.3 in HEL, including contact residues that are shared with the peptides (Gly117, Asp119, Gln121 and Arg125). The shared residues lie on a ridge that fits into a groove in the D1.3 paratope (Fig. 4B). These residues make important contacts with the Ab paratope indicating that D1.3 selected peptides that shared features in common with its cognate epitope on HEL. Importantly, the cross-reactive peptides mimic only a portion (41%; see Supplemental Table 7) of the full D1.3 epitope that protrudes from the epitope's surface (Fig. 4A) and binds to a depression in a subregion of the full paratope (Fig. 4B and Supplemental Figure 5A).

PDPL screenings with a second anti-HEL MAb, HyHEL-5 (Tables 2, 4A and 4B) also yielded HyHEL-5-specific peptides whose consensus residues matched MAb-contacting residues in HEL (Table 4A). For example, HyHEL-5p (TCVGSRGDFMPHPRCG), Arg6, Gly7 and Arg14 correspond to contact residues Arg45, Gly67 and Arg68 in the native HEL epitope (Cohen et al., 1996). As seen for D1.3, shared residues between the HyHEL-5 epitope and HyHEL-5p peptide form a ridge that protrudes from the surface of the protein but does not cover the entire epitope (Fig. 4C); this ridge fits into a depression in the HyHEL5 paratope (Fig. 4D and Supplemental Figure 5B).

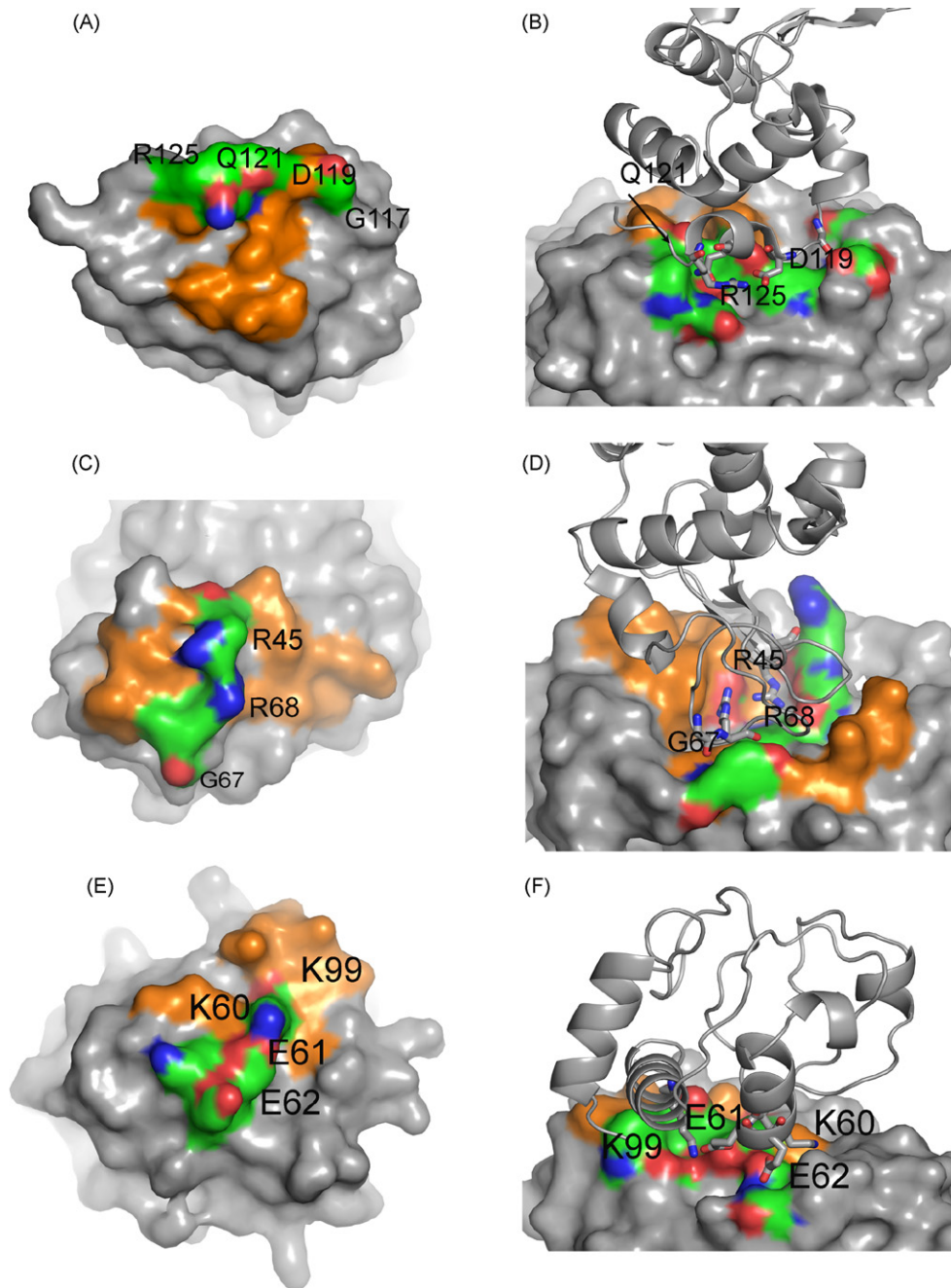


Fig. 4. Surface topology of the D1.3 (A and B), HyHEL-5 (C and D), and E8 (E and F) epitopes and paratopes. Surface of the Ag epitopes (A, C and E) with residues shared between the epitope and the selected peptides shown in green, red and blue, with the remainder of the epitope shown in orange. Oxygen and nitrogen atoms that make direct contacts with Ab are shown in red and blue, respectively. Surface representation of the Ab paratopes (B, D and F) in complex with Ag (ribbon and epitopes in α -carbon backbone representation). Residues in the paratope that contact residues shared between the epitope and the peptide are shown in green with oxygen and nitrogen contacts in red and blue; the remainder of the paratope is orange. Shared residues in the epitopes are labeled and shown as ball-and-sticks. The paratopes by themselves are shown in Supplemental Figure 5. Images were generated using Protein Data Bank file 1FDL for the HEL:D1.3 complex, 1YQV for the HEL:HyHEL complex and 1WEJ for the CytC:E8 complex (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.).

Table 4

A sequence comparison of the cross-reactive peptides for MAb D1.3, HyHEL-5 and E8 with their cognate epitope(s) on HEL (A) and CytC (B).

A Peptide	Peptide Residues in Common with Cognate HEL Epitope
D1.3p-E ^a	<u>SQPN</u> *C*D* <u>G</u> *LVC*NR*WH
D1.3p-C ^b	TCYP <u>Q</u> TSTTPPH <u>C</u> D
HyHEL-5p ^c	TCVGS <u>R</u> GDFMPHP <u>R</u> CG
KVFGRCLEAAAMKRHGLDNYRYSGLGNVCAAKFESNFNTQATN(<u>R</u> ₁₄₅)	
NTDGGTDYGLQINSRWWCND(<u>G</u> ₆₇)(<u>R</u> ₆₈)TPGSRNLNIPCSALLSS-	
DITASVNCARKIVSDGNGMNAVAVNRNCR(<u>G</u> ₁₁₇)(<u>T</u> ₁₁₉)(<u>V</u> ₁₂₁)(<u>AWIR</u> ₁₂₅)(<u>GCRL</u>	
B Peptide	Peptide Residues in Common with Cognate CytC Epitope
E8p ^d	MC*P*P <u>D</u> * <u>D</u> *T <u>R</u> KL* <u>C</u> *I
GDVEKGKKIVFQKCAQCHTVEKGGKHKTGPNLHGLFGRKTGQAPGFTYTANK-	
NKGITW(<u>K</u> ₆₀)(<u>E</u> ₆₁)(<u>E</u> ₆₂)TLMEYLENPKKYTPGKMFAGIKKKTERED-	
LIAYL(<u>K</u> ₉₉)KATNE	

^a Consensus residues from the PDPL screenings are underlined. CBRs in common between D1.3p-E and the cognate HEL epitope are shown in bold with the amino acid position shown in brackets within the HEL amino acid sequence (assuming Asn substitutes for Gln). *Residues determined to be critical or important for binding by Ala-scan (see Fig. 3) or binding in competition ELISA with Ala-replacement peptides (see Table 5).

^b Consensus residues for D1.3p-C are underlined. Consensus residues in common with HEL CBRs are in bold.

^c Consensus residues fixed in the sublibrary for HyHEL-5 are underlined. Residues in common between HyHEL-5p and its cognate HEL epitope are in bold italics.

^d Consensus residues for E8p are underlined. Consensus residues in common with known contact residues of the cognate CytC epitope are in bold (assuming Asp and Glu are interchangeable).

Selection of residues present in the native CytC epitope was also observed for the E8p peptide (MCPDDTKRLCI; consensus residues in bold; Table 4B). Assuming that Asp can substitute for Glu, Asp5 and Asp6 on the E8p sequence are contact residues in common with the native CytC epitope, which comprises three main segments (residues 33–39, 56–66 and 96–104) as well as two minor ones (Mylvaganam et al., 1998) (Fig. 4E). In addition, Lys8, a consensus residue found in peptides selected from the E8 library screenings, may correspond to either Lys 60 or Lys99, both of which are contact residues in the E8 epitope of CytC. As with the D3.1 and HyHEL5 peptides, E8p mimics a ridge-like portion of the E8 epitope that interacts with a wall in a depression in the E8 paratope (Fig. 4F and Supplemental Figure 5C).

In summary, all three MAbs selected peptides bearing CBRs that are shared with contact residues on their respective cognate epitopes. In all cases, these residues modeled onto the surface of the cognate Ag produced a ridge that is contacted by a groove or depression in the Ab paratope. Moreover, in all three cases, the shared residues are restricted to a subregion within the complete epitope where shared residues were calculated to comprise ~40–50% of the original epitope (Krissinel and Henrick, 2007) (see Supplemental Table 7).

4. Discussion

The HEL:D1.3 complex represents one of the most highly characterized protein–protein interactions (Amit et al., 1986; Fischmann et al., 1991), and thus, it is particularly well-suited for studying the concept of “epitope-targeted vaccines”. MAb D1.3 has only five somatic mutations from its germline genes (only one of which, V_LTyr50, directly contacts HEL) (England et al., 1999). An idiotypic network established for D1.3 by Goldbaum et al. (1997) demonstrated that an anti-(D1.3) idiotypic MAb elicited anti-anti-idiotypic MAbs that bound to both HEL and the anti-(D1.3) idiotypic MAb via many of the same contacts as D1.3. Remarkably, the V-

gene usage of the anti-anti-idiotypic MAbs was very similar to that of D1.3. Thus, the network suggested that the anti-(D1.3) idiotypic MAb was mimicking the D1.3 epitope on HEL, and proved that a mimic can elicit ‘D1.3-like’ Ab specificities.

In our study, however, peptides selected by D1.3 MAb (which could be considered the equivalents of the anti-(D1.3) idiotypic MAb of Goldbaum et al., 1997) failed to elicit Abs that could bind HEL. Extensive characterization of one of the peptides (D1.3p-E) suggested that the lack of immunogenic mimicry was not due to poor affinity of the peptide with its MAb. Interestingly, the peptides selected by MAb D1.3 bore CBRs in common with Ab-contacting residues in the native epitope that are important for the interaction between D1.3 and HEL. Analysis of these shared residues on the surface of HEL revealed that they produce a ridge that is contacted by a groove or depression in the Ab paratope. The shared residues, however, did not cover the complete epitope, which may explain, in part, the failure of these peptides to act as immunogenic mimics. A similar situation was found for the peptide ligands of MAbs HyHEL-5 and E8.

It is also possible that CBRs of peptides we identified for all three MAbs, as being shared with contact residues in the corresponding epitopes on HEL and CytC, are not truly “mimicking” those contact residues. The peptides’ precise mechanism of binding can only be determined via crystallographic analysis in complex with Fab (our attempts to produce a co-crystal of the D1.3p-E peptide in complex with D1.3 Fab have so far failed.) In previous work, we showed that the Ab-binding mechanisms of a cross-reactive peptide and its cognate discontinuous protein epitope can be entirely different (Saphire et al., 2007). Given this caveat, our findings for three well-characterized discontinuous epitopes show that cross-reactive peptides selected from PDPLs, even if optimized for Ab affinity, are not effective immunogenic mimics.

The central paradigm of Ab–Ag interactions is that the three-dimensional structure of the six hypervariable loops forming the Ab paratope binds to a chemically and structurally complementary surface on Ag (Cohen et al., 1996; Wilson and Stanfield, 1994). Interestingly, it has been shown that all of the murine and human complementarity-determining regions (CDRs) of heavy chains and kappa light chains, except for the heavy chain CDR3, include conserved residues and assume a limited set of lengths, resulting in defined “canonical” structures of the loops (Chothia et al., 1989). In some cases the combination of canonical structures comprising an Ab paratope can correlate with the type of Ag (i.e., protein versus peptide versus hapten) to which it binds (Almagro, 2004; Collis et al., 2003; Lara-Ochoa et al., 1996; Sundberg and Mariuzza, 2002; Vargas-Madrado et al., 1995). Anti-protein Abs are typically flat with small protuberances and depressions that form a relatively large complementary interface with protein, whereas the binding sites of anti-peptide Abs, tend to be groove-like, and peptides frequently bury themselves as beta turns within those grooves (MacCallum et al., 1996; Wilson and Stanfield, 1994). These differences in paratope topology of anti-protein versus anti-peptide Abs, and the relatively small size of peptide epitopes, represent inherent weaknesses in peptide-based vaccines (Van Regenmortel, 2009), which are experimentally demonstrated here. The one successful example of immunogenic mimicry for which side-by-side co-crystal structures are available (an anti-carbohydrate Ab bound to both peptide and cognate oligosaccharide epitopes (Borrelli et al., 2008), shows that while the peptide did not recapitulate all of the contacts made between the Ab and the cognate epitope, it fit well into the cognate epitope’s footprint, and the affinity of binding to peptide and oligosaccharide were similar (Vyas et al., 2003). Thus, at least for peptide mimicry of carbohydrate, perfect mimicry of Ab contacts may not be as important as recapitulating the overall topology of the bound cognate epitope, and perhaps, its affinity. This may be also a requirement for peptide mimics of

discontinuous protein epitopes, but one that may be difficult to satisfy.

Nonetheless, it has been speculated that changes in immunization approaches could produce cross-reactive Abs (van Houten and Scott, 2005; Zwick et al., 2001a,b). Others have used a prime-boost immunization strategy in which mice are primed with whole Ag and then boosted with peptide (Beenhouwer et al., 2002; Borrelli et al., 2008; Davis et al., 1997). The rationale for this approach is that if the cognate Ag primes the immune response, including against the targeted epitope, then cross-reactive peptide(s) can be used to amplify the targeted Ab response. Our attempts to prime with HEL and boost with recombinant D1.3p-E phage, failed to yield cross-reactive Abs (data not shown), suggesting that the D1.3 specificity is infrequent in the primary immune response to HEL. This is further evidenced by the fact that all anti-HEL sera tested failed to bind the D1.3p-E peptide. These results suggest that a significant (i.e., detectable) primary response against the epitope of interest is likely required for a prime-boost immunization approach to work, and help to explain the limited success met by this strategy so far. Indeed there is evidence of immunodominant gene usage, which probably is recruited in the primary response, in protective Ab responses against pathogens such as human cytomegalovirus (McLean et al., 2005), rotavirus (Weitkamp et al., 2003), *Streptococcus pneumoniae* (Zhou et al., 2002) and *Haemophilus influenzae* (Adderson et al., 1991).

The successful development of vaccines targeting discontinuous epitopes still evades researchers involved in vaccine design. Techniques including rational design, occlusion of undesired epitopes (Nara and Garrity, 1998; Pantophlet et al., 2003) and the selection of peptide mimics from PDPLs (Saphire et al., 2007) have all failed to effectively produce Ab responses having the desired specificity. The work presented here is especially meaningful for vaccine development, since it indicates that partial overlapping of contact residues and a reasonable affinity of interaction do not directly translate into immunogenic mimicry, even within an inbred animal model system in which species differences in the Ab repertoires are not an issue (as has been the case for anti-HIV NtAbs, for example, Menendez et al., 2008; Saphire et al., 2007). Our findings suggest that, in general, direct immunization with peptide mimics of discontinuous epitopes is not a feasible approach to the development of epitope-targeted vaccines, and suggest approaches that take into account the topology of discontinuous epitopes on protein Ags.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2009.10.015.

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Supplemental Table 1: Sequences of synthetic peptide used in this work.

Peptide Name	Peptide Sequence	Mol. Wt.
D1.3p-C	NH ₃ ⁺ -TCYPQTSTTPPHCD <u>PAEG</u> GK -CONH ₂ [†]	2089.3
D1.3p-E	NH ₃ ⁺ -ASQPNCDGLVCNRWH <u>PAEG</u> GK -CONH ₂	2238.5
E8p-WT [#]	NH ₃ ⁺ -MCP P DDTKRLC <u>IAAE</u> -CONH ₂	1662.0
E8p-M1A	NH ₃ ⁺ - A C P PDDTKRLC <u>IAAE</u> -CONH ₂	ND [*]
E8p-C2S	NH ₃ ⁺ -M S P P DDTKRLC <u>IAAE</u> -CONH ₂	ND
E8p-P3A	NH ₃ ⁺ -MC A PDDTKRLC <u>IAAE</u> -CONH ₂	ND
E8p-P4A	NH ₃ ⁺ -MCP A DDTKRLC <u>IAAE</u> -CONH ₂	ND
E8p-D5A	NH ₃ ⁺ -MCP P A DDTKRLC <u>IAAE</u> -CONH ₂	ND
E8p-D6A	NH ₃ ⁺ -MCP P D A TKRLC <u>IAAE</u> -CONH ₂	ND
E8p-T7A	NH ₃ ⁺ -MCP P DD A KRLC <u>IAAE</u> -CONH ₂	ND
E8-K8A	ND [*]	-
E8p-R9A	NH ₃ ⁺ -MCP P DDTK A LC <u>IAAE</u> -CONH ₂	ND
E8p-L10A	NH ₃ ⁺ -MCP P DDTKR A C <u>IAAE</u> -CONH ₂	ND
E8p-C11S	NH ₃ ⁺ -M S P P DDTKRL S <u>IAAE</u> -CONH ₂	ND
E8p-I12A	ND	-
E8p-A13G	NH ₃ ⁺ -MCP P DDTKRLC <u>I</u> G <u>AE</u> -CONH ₂	ND
E8p-A14G	ND	-
E8p-E15A	ND	-

[†]The underlined AAE sequence corresponds to fixed amino acids flanking the C-terminal side of phage displayed peptides. Bolded GK sequence was added for spacing and conjugation, respectively.

[#]WT; wild-type sequence. All other E8p peptides have amino acid replacements where indicated in bold.

^{*}ND; not done. These peptides could not be synthesized, or the molecular weight was not determined.

Supplemental Table 2: Screening of primary PDPLs and sublibraries with biotinylated MAbs D1.3, Hy-HEL 5 and E8.

MAb & Library type	D1.3 primary PDPLs	D1.3 sublibrary E	D1.3 sublibrary C	HyHEL-5 primary PDPLs	HyHEL-5 sublibrary A	E8 primary PDPLs
Round 1	solid-phase plates IgG	solid-phase beads Fab	solid-phase beads Fab	in-solution beads IgG 100nM	in-solution beads IgG 10 and 100 nM	solid-phase plates IgG
Round 2	in-solution beads IgG 100 nM	in-solution beads Fab 10, 1 and 0.1 nM	in-solution beads Fab 10, 1 and 0.1 nM	in-solution beads IgG 50 nM	in-solution beads IgG 5 and 0.1 nM	solid-phase beads and in-solution beads IgG and Fab 50, 10 and 1 nM
Round 3	in-solution beads IgG 50 nM	in-solution beads Fab 1nM	in-solution beads Fab 10, 1 and 0.1 nM	in-solution beads IgG 10 nM	in-solution beads IgG 0.1 nM and 50 pM	in-solution beads Fab 50, 10, 1 and 0.1 nM
Round 4	in-solution beads IgG 5 nM	ND	ND	ND	ND	ND

Supplemental Table 3: Epitope mapping of HEL with anti-HEL mouse serum.

a.a. # ^a	Blot Spot	HEL peptides ^b	serum reactivity ^c	a.a.#	Blot Spot	HEL peptides	serum reactivity
1-10	A1	KVFGRSELA		61-70	D1	RWWSNDGRTP	
3-12	A2	FGRSELAAM		63-72	D2	WSNDGRTPGS	
5-14	A3	RSELAAMKR		65-74	D3	NDGRTPGSRN	
7-16	A4	ELAAAMKRHG		67-76	D4	GRTPGSRNLS	
9-18	A5	AAAMKRHGLD		69-78	D5	TPGSRNLSNI	
11-20	A6	AMKRHGLDNY		71-80	D6	GSRNLSNIPS	
13-22	A7	KRHGLDNYRG		73-82	D7	RNLSNIPSSA	
15-24	A8	HGLDNYRGYS		75-84	D8	LSNIPSSALL	
17-26	A9	LDNYRGYSLG		77-86	D9	NIPSSALLSS	
19-28	A10	NYRGYSLGNW		79-88	D10	PSSALLSSDI	
21-30	B1	RGYSLGNWVS		81-90	E1	SALLSSDITA	
23-32	B2	YSLGNWVSAK		83-92	E2	LLSSDITASV	
25-34	B3	LGNWVSAAKF		85-94	E3	SSDITASVNS	
27-36	B4	NWVSAAKFE		87-96	E4	DITASVNSAK	
29-38	B5	VSAAKFESNF		89-98	E5	TASVNSAKKI	
31-40	B6	AAKFESNFNT		91-100	E6	SVNSAKKIVS	
33-42	B7	KFESNFNTQA		93-102	E7	NSAKKIVSDG	
35-44	B8	ESNFNTQATN		95-104	E8	AKKIVSDGNG	
37-46	B9	NFNTQATNRN		97-106	E9	KIVSDGNGMN	
39-48	B10	NTQATNRNTD		99-108	E10	VSDGNGMNAW	
41-50	C1	QATNRNTDGS		101-110	F1	DGNGMNAWVA	
43-52	C2	TNRNTDGSTD		103-112	F2	NGMNAWVAWR	
45-54	C3	RNTDGSTDYG		105-114	F3	MNAWVAWRNR	
47-56	C4	TDGSTDYGIL		107-116	F4	AWVAWRNRSK	
49-58	C5	GSTDYGILQI		109-118	F5	VAWRNRSKGT	
51-60	C6	TDYGILQINS		111-120	F6	WRNRSKGTDV	
53-62	C7	YGILQINSRW		113-122	F7	NRSKGTDVQA	
55-64	C8	ILQINSRWWS		115-124	F8	SKGTDVQAWI	
57-66	C9	QINSRWWSND		117-126	F9	GTDVQAWIRG	
59-68	C10	NSRWWSNDGR		119-127	F10	DVQAWIRGSR	
				120-129	G1	VQAWIRGSRL	

Legend:

No binding	
Weak binding	
Medium binding	
Strong binding	

^a HEL amino acid number (a.a.#).

^b HEL Cys residues replaced by Ser in the overlapping peptides are in bold.

^c Anti-HEL sera were diluted 1:100 to probe the HEL peptide membrane. Sera from an unimmunized mouse as well as from an OVA-immunized mouse (also diluted 1:100) did not bind to any of the HEL peptides. The secondary Ab alone, HRP-conjugated-goat- (anti-mouse-IgG-Fc)-Ab, diluted 1:2500, did not bind to any of the peptides either.

Supplemental Table 4: Sequences and ELISA analysis for phage clones enriched from the NNK-doped D1.3p-E phage sublibrary.

Clone	Peptide Sequence ^a	10nM D1.3 Fab	1nM D1.3 IgG ^c
4A.9	PRPNCDGSVCIRWH	1.09	1.36
4B.17	SQPNCDGLVCIRWH	0.91	1.31
3C.15	SQPNCDGLVCVRWH	0.81	1.34
4B.12	PAPNCDGGVCVRWH	0.80	1.33
4A.5	LQPNCDGHKCVRWH	0.79	1.34
3A.1	PQPNCDGLVCVRWH	0.78	1.37
3B.6	LPPNCDGPVCCRHT	0.76	1.26
2B.5	SQPNCDGLVCNSWH	0.75	1.29
	HEL	0.73	1.17
3B.4	PQPNCDGLVCNRWH	0.72	1.27
3B.7	RQPNCDGLVCNRRH	0.72	1.27
3B.10	RQPNCDGLVCNRRH	0.71	1.32
3C.18	KRPNCDGGTCNRWH	0.71	1.30
4A.3	SLPNCDGLVCNRWH	0.71	1.25
2A.15	PQPNCDGTVCARWH	0.70	1.26
3A.7	ATPNCDGLVCSRWH	0.65	1.33
4C.10	AAPNCDGLVCNRWH	0.61	1.24
4A.7	PLPNCDGGVCNRWH	0.60	1.20
D1.3p-E	SQPNCDGLVCNRWH	0.60^b	1.18
2A.11	VTPNCDGTFCNRWH	0.59	1.34
3C.13	SIPNCDGLVCYRWH	0.59	1.34
3A.9	LLPNCDGLVCNRLH	0.38	1.11
2A.1	PQANCDGLVCERWH	0.36	1.14
4A.2	RTPNCDGQVCNRWH	0.25	1.31
	f88 phage	0.23	0.03
2A.12	PDPNCDGLVCNRAP	0.16	0.04
3A.14	STPHCDTLFCNRWR	0.16	0.04
2A.9	SAPNCDGLVCSRWS	0.14	0.10

3B.5	HP P NCHPQNC T QL	0.08	0.04
3C.4	S Q P NCDHPMC N P W H	0.04	0.18

^a Residues in common with the D1.3p-E peptide are in bold.

^b ELISA binding signals for the wt D1.3p-E phage are in bold.

^cHRP-conjugated-goat-(anti-mouse-IgG-H+L)-Ab was used as the secondary Ab for detecting D1.3 Fab and D1.3 IgG.

Supplemental Table 5: Sequences and ELISA analysis for phage clones enriched from the HyHEL-5p phage sublibrary.

Clone Name^a	Peptide Sequence	ELISA Signal O.D. 405-490
A9.2	TCVGSRGDFMPHPRCG	0.82
A7.1	ACVGHARGPT- - - -RCGL	0.52
Sublibrary^b	XCVGXRGXXXXXXXXRCX	--
3'.3-2	QCVGNRGDWMDFPRCR	0.91
3.3b-4	KCVGNRGQMHAGTRCF	0.90
3.3a-3	SCVGNRGNPHXPXSRCI	0.90
3'.3-3	QCVGSRGDFHQGSRCV	0.86
3'.3-4	ACVGHRGSHLPPSRCL	0.76
3.3b-1	MCVGNRGDFHHGQRCL	0.71
3'.3-1	QCVGNRGDGHWTFRCR	0.69
3.3a-4	NCVGNRGNALPTSRL	0.68
3.3a-2	TCVGNRGTFHLGNRCA	0.67
2'.3-3	KCVGQRGSA PGPYRCF	0.66
2.3b-4	DCVGNRG PAPGHLRCS	0.28
2.3a-5	NCVGNRGYWHDAARCT	0.19
2.3a-3	DCVGNRGSHFISTRCH	0.17
2'.3-2	ECVGNRGNWHAVTRCT	0.12
2.3b-3	NCVGRRGNFHYFSRCL	0.11
2.3a-4	ACVGNRGTF LVP SR CM	0.08
2.3a-2	SCVGNRGLFLPPGRCQ	0.07
2.3b-1	MCVGP RGLEPDSARCL	0.05
2.3b-2	SCVGNRGAWLSRPRCI	0.01
2.3b-5	ECVGNRGPGMLGTRCN	0.01
2'.3-1	ACVGNRGDIPSGHRCF	0.01

^a Clones are numbered according to the round from which they were selected: clones labeled 3 or 3' were from round 3 and clone labeled 2 or 2' were from

round 2. Clones labeled 2 or 3 were screened using a starting concentration of 100 nM HyHEL-5 and clones labeled 2' or 3' used a starting concentration of 10 nM.

^b The sub-library was made from the parent clone A9.2, with residues in bold being fixed and all other residues variegated.

Supplemental Table 6: Residues comprising the epitope and paratope from crystal structures of D1.3 and HyHEL-5 in complex with HEL and E8 in complex with CytC.

	Paratope				Epitope	
	Residues that contact residues shared between epitope and peptide ^a		Whole Paratope ^b			
MAb	V _L	V _H	V _L	V _H	Residues shared between epitope and peptide ^c	Whole Epitope ^d
D1.3^e Fischmann et al., 1991	Y32, F91, W92, S93	G31, W52, G53, D54, Y101	Y32, Y50, T53, F91, W92, S93	G31, W52, G53, D54, D100, Y101, R102	G117(C ^α , O), D119(C, C ^α , C ^β , C ^γ , O ^{δ1}), Q121(C ^β , C ^γ , C ^δ , N, N ^{ε2} , O ^{ε1}), R125(C ^γ , C ^δ)	D18, N19, G22, S24, N27, G117, T118, D119, V120, Q121, I124, R125
HyHEL-5^e Cohen et al., 1996	W91, G92, R93	E50, Y97	N31, Y32, D50, W91, G92, R93	W33, W47, Q50, L52, S54, G55, S56, T57, N58, G95, N96, Y97	R45 (O, N ^{η2} , N ^ε , N ^γ), G67(O), R68(N ^{η1})	Q41, T43, N44, R45, N46, D48, G49, Y53, G67, R68, T69, P70, L84
E8^e Mylvaganam et al., 1998.	Y32, F91, W92	Y33, R50, N57, D100, Y101	H30, N31, Y32, N50, F91, W92	Y33, R50, N57, D100, Y101	K60 (C ^δ , C ^β), E61(O ^{ε1} , O ^{ε2} , C ^δ), E62(O ^{ε1} , O ^{ε2} , C ^δ), K99(C, O, C ^α , C ^γ , C ^δ , C ^ε , N ^ζ)	G37, F36, K60, E61, E62, A96, K99, K100, N103, E104,

^a Shown in green on Figure 4 and Supplemental Figure 4

^b Shown in orange on Figure 4 and Supplemental Figure 4.

^c Atoms that contact the paratope are shown in brackets (these are shown in green on Figure 4 and Supplemental Figure 4).

^d Shown in orange on Figure 4 and Supplemental Figure 4.

^e PDB files: HEL/D1.3, 1FDL; HEL/HyHEL5, 1YQV; E8/CytC, 1WEJ.

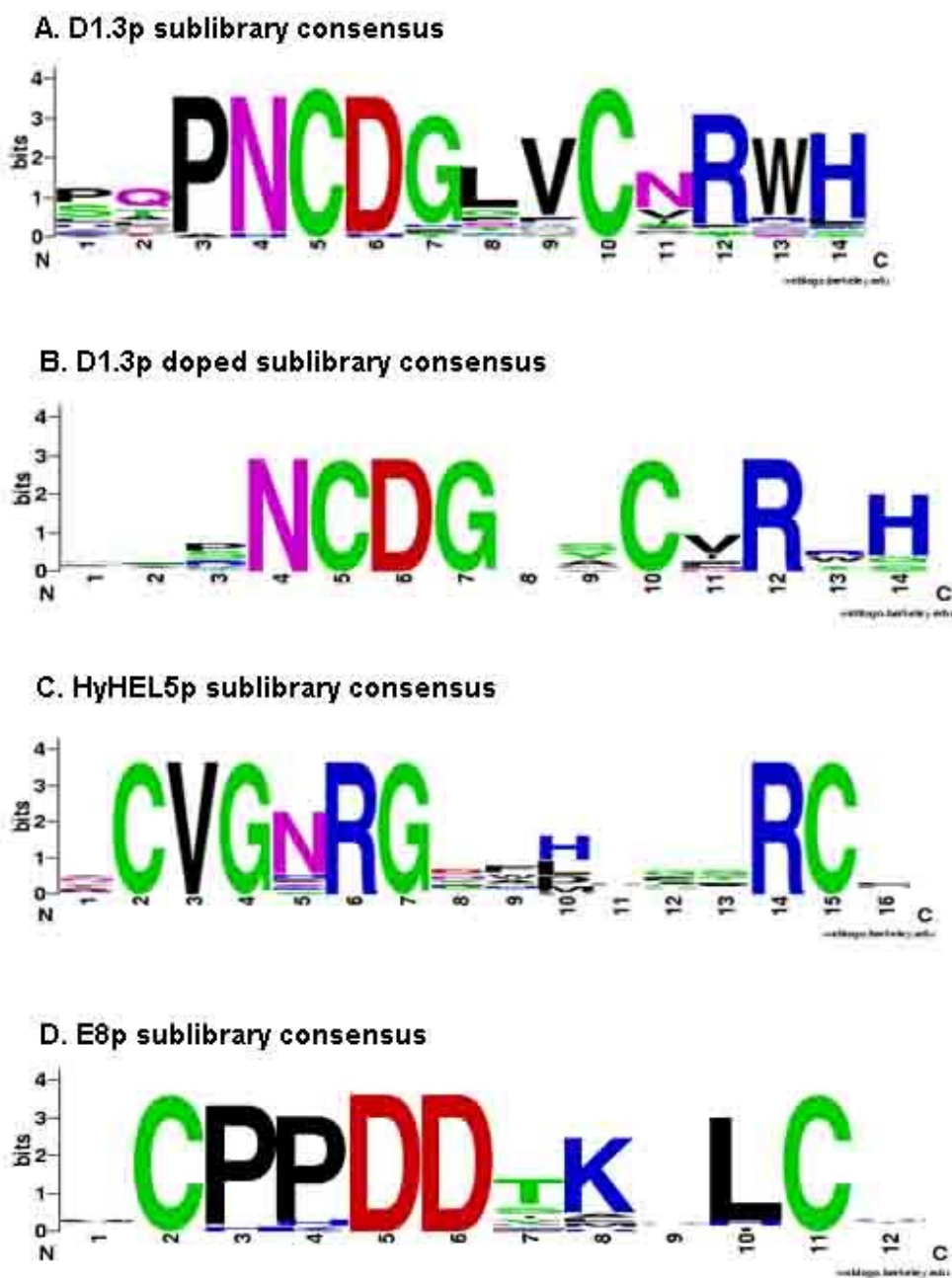
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Supplemental Table 7: Percentage of epitope and paratope occupied by shared residues on peptides compared to their Ab/antigen complexes

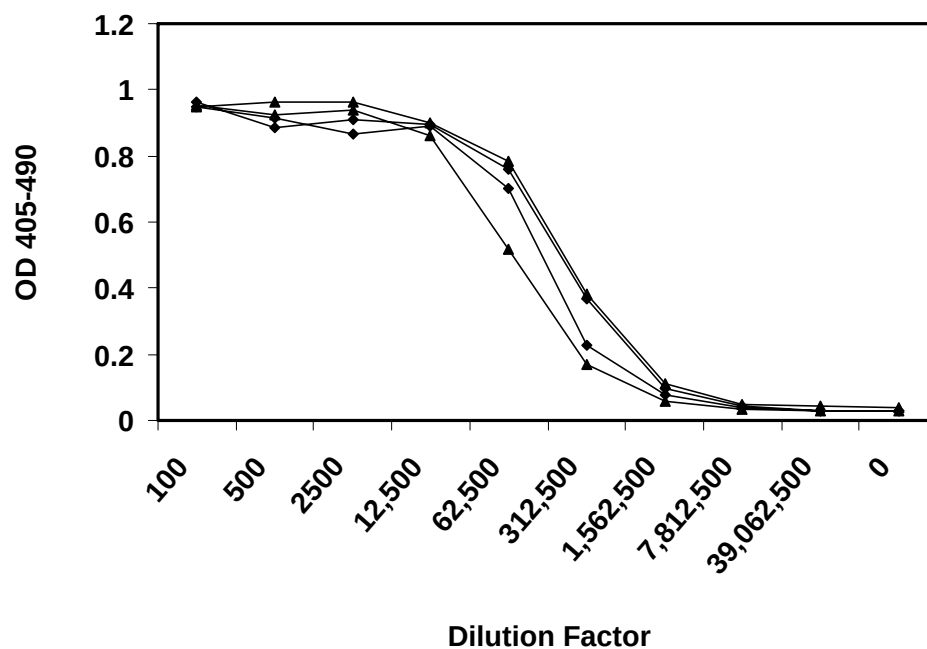
Buried surface area (Å²)^a						
D1.3 and HEL Ab/antigen complex^b						
HEL D1.3 epitope			HEL residues shared w/ peptide			
buried by H	buried by L	total	buried by H	buried by L	total	% of epitope
357.8	317.6	675.4	144.8	134.2	279	41%
D1.3 paratope			D1.3 residues that contact residues shared between HEL and peptide			
H chain	L chain	total	H chain	L chain	total	% of paratope
379.3	268.9	648.2	191.5	161.2	352.7	54%
HyHEL5 and HEL Ab/antigen complex^b						
HEL HyHEL5 epitope			HEL residues shared w/ peptide			
buried by H	buried by L	total	buried by H	buried by L	total	% of epitope
474.6	382.5	857.1	222.4	217.3	439.7	51%
HyHEL5 paratope			HyHEL5 residues that contact residues shared between HEL and peptide			
H chain	L chain	total	H chain	L chain	total	% of paratope
508.1	376.2	884.3	116.4	237.7	354.1	40%
E8 and CytC Ab/antigen complex^b						
CytC E8 epitope			CytC residues shared w/ peptide			
buried by H	buried by L	total	buried by H	buried by L	total	% of epitope
295.3	346.8	642.1	216.3	87.9	304.2	47%
E8 paratope			E8 residues that contact residues shared between CytC epitope and peptide			
H chain	L chain	total	H chain	L chain	total	% of paratope
280.1	334.4	614.5	200.9	171.6	372.5	61%

^a Calculated using EBI PISA program (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) using a 1.4 Å probe radius (Krissinel and Henrick, 2007).

^b PDB files: HEL/D1.3, 1FDL; HEL/HyHEL5, 1YQV; E8/CytC, 1WEJ.

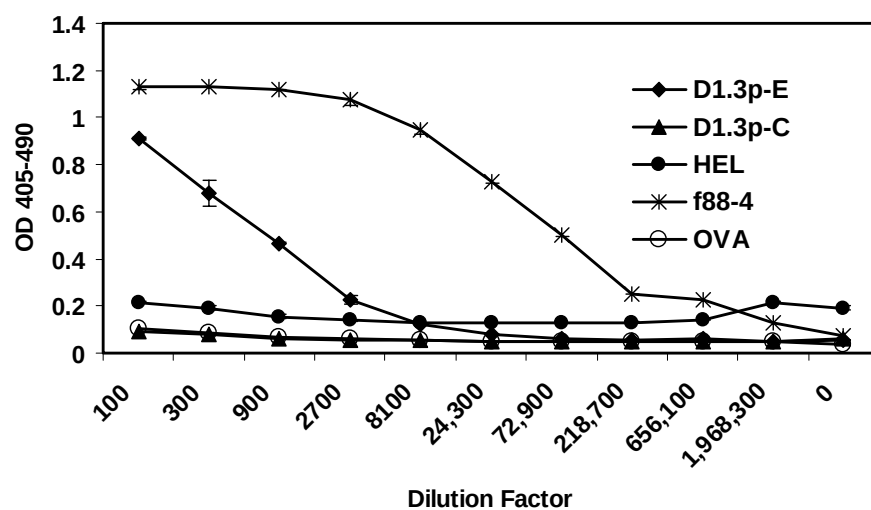


Supplemental Figure 1: Graphical consensus plots for peptides selected from phage-displayed peptide library screenings.

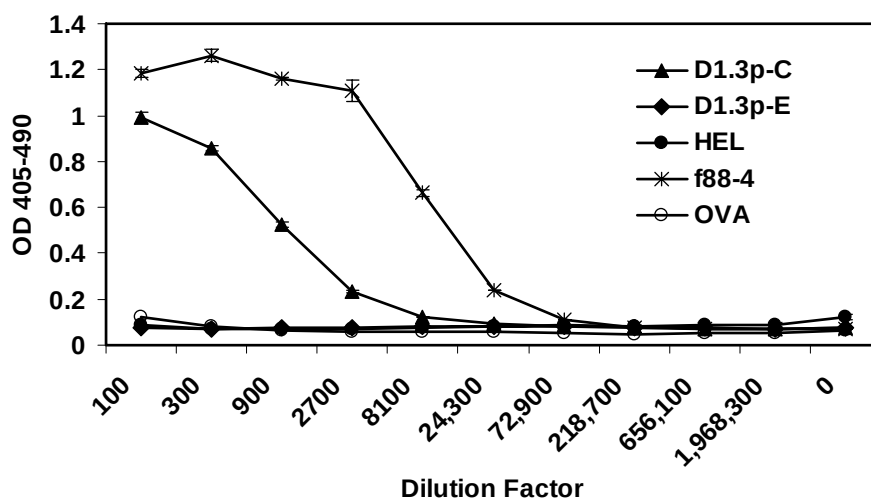


Supplemental Figure 2. Titration ELISA for sera from four mice immunized four times with 50 μ g HEL and the adjuvant Adjuvax.

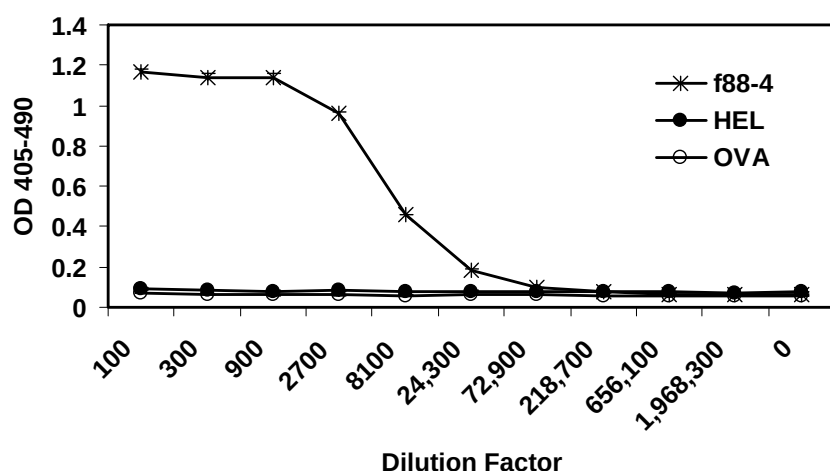
A. D1.3p-E phage immunization



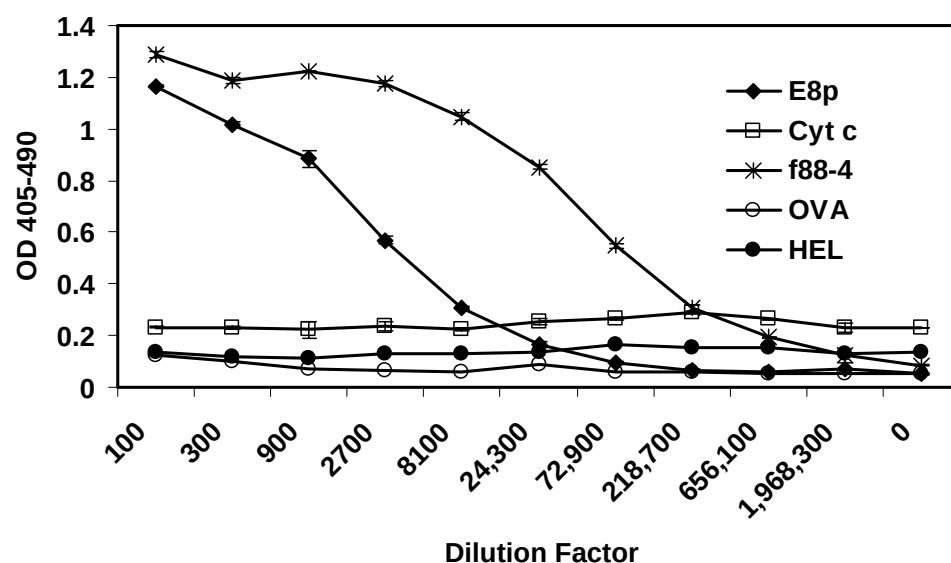
B. D1.3p-C phage immunization



C. HyHEL5p phage immunization

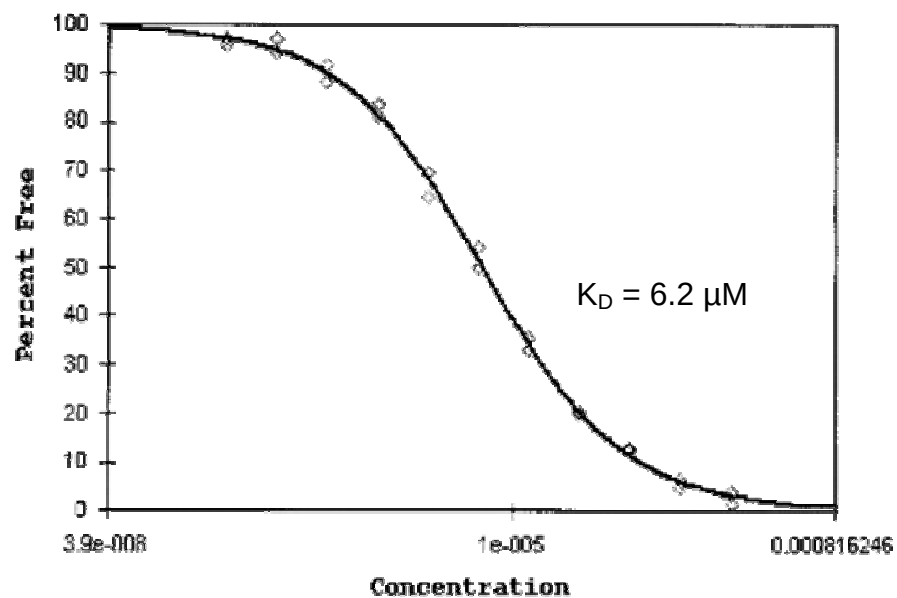


D. E8p phage immunization

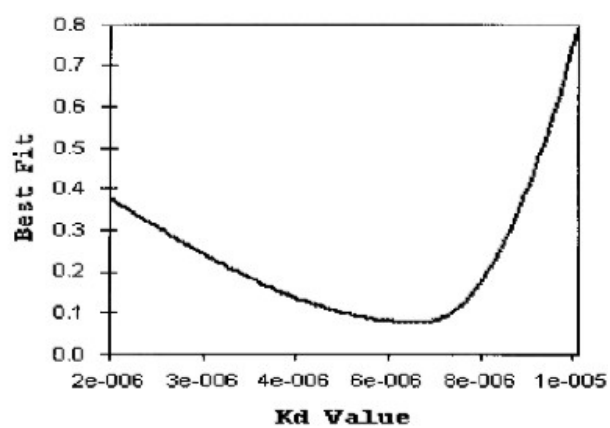


Supplemental Figure 3. Serum titration curves from immunizations with phage displaying Ab-binding peptides D1.3p-E (**A**), D1.3p-C (**B**), and HyHEL5p (**C**) and E8p (**D**). The HyHEL5p peptide could not be synthesized, thus peptide reactivity was not assessed for sera from immunizations with the HyHEL5p phage clone.

A

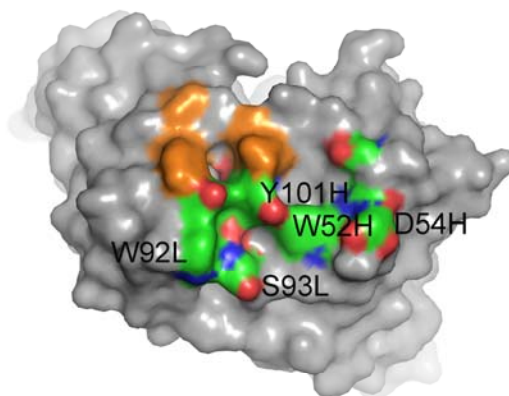


B

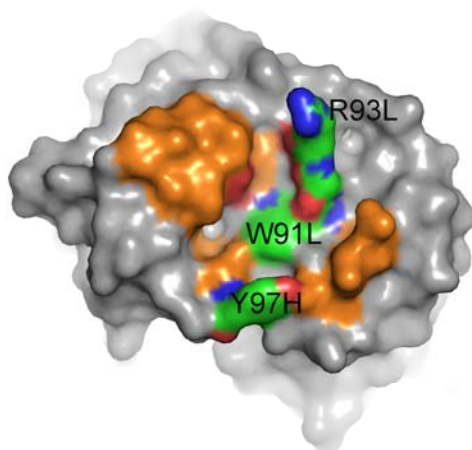


Supplemental Figure 4. Analysis of KinExA data for D1.3 Fab and synthetic peptide, D1.3p-C. (A) K_D curve, and, (B) 95% confidence interval. The experiment was conducted in duplicate.

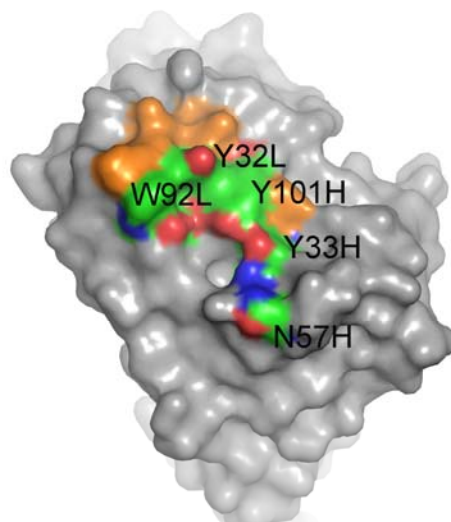
A. D1.3



B. HyHEL5



C. E8



Supplemental Figure 5: Surface topology of paratopes for D1.3 (A), HyHEL-5 (B) and E8 (C) MAbs. The whole epitope is shown in orange and the residues that contact residues that are shared between the peptide and the epitope are shown in green and colored by atom. For reference, some of these residues have been highlighted on the figure; "L" and "H" refer to light-chain and heavy-chain residues, respectively