Structural and biochemical characterization of the vaccinia virus envelope protein D8
and its recognition by the antibody LA5

Running title: Structure of the VACV D8/LA5 Fab complex

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Abstract

Smallpox vaccine is considered a gold standard of vaccines, as it is the only one that has led to the complete eradication of an infectious disease from the human population. B cell responses are critical for the protective immunity induced by the vaccine, yet their targeted epitopes recognized in humans remain poorly described. Here we describe the biochemical and structural characterization of one of the immunodominant vaccinia virus (VACV) antigens D8 and its binding to the monoclonal antibody LA5, which is capable of neutralizing VACV in the presence of complement. Full length D8 ectodomain was found to form a tetramer. We determined the crystal structure of the LA5 Fab - monomeric D8 complex at a resolution of 2.1 Å, as well as the unliganded structures of D8 and LA5-Fab at resolutions of 1.42 Å and 1.6 Å, respectively. D8 features a carbonic anhydrase (CAH) fold that has evolved to bind to the glycosaminoglycan (GAG) chondroitin sulfate (CS) on host cells. The central positively charged crevice of D8 was predicted as the CS binding site by automated docking experiments. Furthermore, sequence alignment of various poxvirus D8 orthologs revealed that this crevice is structurally conserved. The D8 epitope is formed by 23 discontinuous residues that are spread across 80% of the D8 protein sequence. Interestingly, LA5 binds with a high affinity lock-and-key mechanism above this crevice with an unusually large antibody – antigen interface, burying 2434 Å² of protein surface.
Introduction

Smallpox, caused by infection with the orthopoxvirus variola, was one of mankind’s greatest plagues, and early vaccine development led to its complete eradication. Although variola is no longer a natural threat to human health, there is fear of its potential use as a biological weapon (2). In addition, the natural zoonotic ability of the related monkeypox virus to infect humans has led to concern that it could evolve into a global pathogen. Sporadic human outbreaks have been reported since 1970 (18, 45), and infected rodents exported to the United States caused a highly publicized human outbreak in 2003 (23, 28, 44).

For a better understanding of poxvirus immunity, we chose VACV as a model, as it is the active component of the vaccine that led to the eradication of smallpox (1, 37). Vaccine-mediated protection against smallpox is largely mediated through the production of highly potent neutralizing antibodies (20). VACV contains approximately twenty-five integral or peripheral membrane proteins (13, 36) from which seventeen have been implicated to function in virus entry and/or fusion (A27, A17, H3, D8, L1, A28, H2, A21, L5, G3, G9, A16, J5, F9, I2, A26 and O3) (36, 37). The entry-fusion complex (EFC), an essential component of VACV-induced cell-to-cell fusion and viral core penetration, is composed of the eight core proteins A16 (42), A21 (55), A28 (49), G3 (26), G9 (26), H2 (48), L5 (54) and O3 (47). Envelope proteins A27 (14, 25) and H3 (31) are involved in cell-adhesion of the intracellular mature virion (MV) to the host cell glycosaminoglycan (GAG) heparan sulfate (HS). Similarly, VACV envelope protein D8, a 32 kDa type 1 membrane protein, binds to cell surface chondroitin sulfate (CS) but not to HS. While VACV infectivity of BALB/c mice is reduced in D8 deletion strains (46), D8 negative virus replicates efficiently in cultured cells (40). This suggests that VACV utilizes several alternate routes of host cell adhesion and infection thus reducing the chances of antibody-mediated neutralization by targeting a single VACV envelope protein. Vaccination of humans with VACV can elicit antibodies against at least four major MV surface antigens (A27, L1, D8, and H3) (6, 17). To date, of the immunodominant VACV envelope proteins (A27, A33, L1, D8, B5 and H3),
only the structures of L1 and A33 have been determined (50, 52), and only one co-crystal
structure exists with a directly neutralizing antibody (L1 + mAb 7D11) (51). Therefore,
information on the structural basis of neutralizing antibodies against the major VACV envelope
proteins is very limited.

Sequence alignments revealed a significant homology of D8 to human carbonic
anhydrases (CAHs, 36% identity over 85% of the D8 ectodomain sequence, (40)) that convert
CO₂ into bicarbonate and back and thus support carbon dioxide removal within the lungs. The
regulatory domain of neural tissue-specific phosphotyrosine-phosphatase receptors (PTPRs,
(38)) also adopts the CAH fold, suggesting that the CAH fold has evolved to carry out multiple
different functions. The CAH catalytic site coordinates a zinc cation that is required for
enzymatic activity. The zinc binding residues are not conserved in D8 (41), and D8 is not
catalytically active for CO₂ conversion (27).

This study focuses on the biochemical and structural characterization of the D8
ectodomain, and its binding to the mouse IgG2α antibody LA5. D8 is an immunodominant
antigen in the smallpox vaccine, and recombinant D8 protein was used successfully, along with
A27 and B5, in a multi-protein vaccination (7). Currently, twelve T cell epitopes for D8 have
been deposited in the immune epitope database (www.immune epitope.org), while no
information about a D8 B cell epitope is available. We show that LA5 monoclonal antibody
(mAb) is neutralizing in presence of the complement. The structural characterization of the
D8/LA5 interface allowed us to define the first discontinuous D8 B cell epitope and its targeting
by the neutralizing antibody LA5. We hypothesize that the LA5 dependent complement
recruitment against D8 competes with binding to CS and, therefore, blocks viral attachment to
the D8 specific host-cell GAG CS.
Materials and Methods

D8 expression and purification. We expressed two forms of recombinant D8 ectodomains; D8 Δ265 (D8 residues 1-264), which includes the D8 unique cysteine at position 262, and D8 Δ262 (residues 1-261) that lacks this unpaired cysteine. D8 Δ265 was used for in-solution characterization and SPR affinity measurements, while D8 Δ262 was engineered to obtain a homogeneous sample for crystallization. The D8 Δ262 DNA fragment was obtained from VACV (strain Acam2000) genomic DNA by amplifying the gene with 5' and 3' primers (5'¬atat cat ATG CGC CTA AAT CAA TGC ATG TCT AAC-3' and 5'-ata tat GGA TCC TTA GTT CAT TGT TTT AAC ACA AAA ATA C-3'), subsequently restricted with NdeI and BamHI and ligated into pET22b for cytosolic expression. For D8 Δ265, the 5' and 3' primers (5'-TTA CCA TGG CGC AAC AAC TAT CTC C-3' and 5'-CTC TCG AGT TAT GAA AAA CAT GTC TCT CTC A-3') were used for PCR amplification and the corresponding DNA fragment was digested with Ncol and Xhol and cloned into pProEX-HTA (Invitrogen). The resulting expression plasmids were transformed into CodonPlus BL21 cells (Agilent). After culture growth to OD of 0.6 D8 expression was induced by induction with 1mM IPTG at 37 °C for 4 h. Cells from typically 1 L were spun down (10 min x 4000 g) and resuspended in 50 ml lysis buffer (50 mM Tris pH8.0, 5 mM EDTA). Cells were sheared with a microfluidizer (3 rounds at 1400 bars, Microfluidics) and crude lysate was clarified by centrifugation (1 h x 50,000 g). Expression resulted in soluble recombinant ectodomains with hexahistidine sequences at the N terminus for D8 Δ265 and at the C-terminus for D8 Δ262 that were further purified by immobilized metal affinity chromatography (IMAC, GE Healthcare). Supernatant was passed through an IMAC column, washed with three column volumes (Vc = 5 ml) of low imidazole buffer (LIB, 50 mM Tris pH 8.0, 300 mM NaCl, 20 mM imidazole). After another wash at 50 mM imidazole, D8 was eluted with the same buffer containing ~ 250 mM imidazole and subsequently dialyzed twice in 10 mM Tris.
pH 8.0, 200 mM NaCl prior to size-exclusion chromatography (SEC) for complex preparation
and/or crystallization.

To analyze D8 oligomerization states, we compared the SEC profile of D8 Δ265 in both
non-reducing (100 mM Tris pH 8.0, 100 mM NaCl) and reducing environments; D8 Δ265 (1
mg/ml) was incubated on ice for 10 min in presence of 10 mM DTT before subjecting to SEC on
Superdex S200 (GL10/300, GE Healthcare) under reducing conditions (100 mM Tris pH 8.5, 100
mM NaCl, 10 mM DTT). For comparison purified D8 Δ265 was also subjected to SEC under
non-reducing conditions, in the absence of DTT. The disulfide-linked oligomeric nature of D8
was analyzed using non-reducing SDS-PAGE, as well as native PAGE.

Antibody sequencing. Total RNA from 5×10⁶ hybridoma cells was isolated using the RNAeasy
Mini kit according to the manufacturer’s instructions (Qiagen). First-strand cDNA was amplified
using OneStep RT-PCR Kit (Qiagen). The cycling profile used for the first strand PCR was 1
cycle of 30 minutes at 50°C and 15 minutes at 95°C; 40 cycles of 30 seconds at 94°C, 30
seconds at 60°C, and 55 seconds at 72°C; and 1 cycle of 10 minutes at 72°C and 4°C cool
down. Second strand PCR was performed using primers for heavy chain IgG2a (5’MsVHE and
3’Cy2c outer) and light chain kappa (5’mVkappa and 3’mCk) (53). PCR products were verified
by gel electrophoresis, with ~500 bp products for heavy chains and ~450 bp products for light
chains. PCR fragments were purified using the QIAquick PCR Purification Kit (Qiagen) and
sequenced (5’-primer extension, using L and H chain specific primers). Sequences only include
V-D-J regions for heavy chains and V-J regions for light chains. Antibody germ line origin was
determined using IMGT’s V-Quest service (11). The LA5-Fab was sequenced as IGKV4-55*01
and IGHV1S127*01.
Viruses. VACV Western Reserve strain (VACV<sub>WR</sub>) stocks were grown on HeLa cells in D-10 media as described (6). The ultraviolet inactivated vaccinia virus (UV-VACV) was prepared as described previously (16)).

Antibodies. Mouse anti-D8 (LA5 clone) and anti-H3 (#41 clone) monoclonal antibodies (mAbs) were derived from a mouse infected with VACV<sub>WR</sub> or immunized with recombinant H3 protein as described (34, 35). For antibody purification, hybridomas were cultured and mAbs were purified from conditioned culture media using recombinant Protein A-Sepharose Fast Flow gel (GE Healthcare) as described (35)(4, 5, 34). The purified antibodies were quantified by the Lowry method using Bovine IgG (Pierce) as a standard, and/or by OD<sub>280</sub> using NanoDrop and calculated using the formula (concentration in mg/ml = 1.35 × OD<sub>280</sub>). The mAbs were stored in aliquots at -80 °C and diluted into PBS immediately prior to use, as needed.

ELISA. Flat-bottomed 96-well microtiter plates (Nunc Maxisorp) were coated overnight at 4°C with 100 µl of 1 µg/ml of recombinant D8 protein (D8) in PBS. Plates were washed (PBS plus 0.2% Tween-20) and blocked (PBS plus 0.5% BSA and 0.2% Tween-20). Purified anti-D8 (LA5 clone) and anti-H3 (#41 clone) mAbs were screened using 3 folds serial dilution ELISA. Secondary antibody: streptavidin-horseradish peroxidase conjugated goat anti-mouse IgG2a (Jackson ImmunoResearch Laboratories, West Grove, PA). The EC<sub>50</sub>, the half-saturation binding concentration of the antibody corresponding to 50% maximum optical density, for anti-D8 monoclonal antibody was determined by sigmoidal dose response nonlinear regression, variable slope (Prism 5.0). For whole vaccinia virus ELISA, plates coated overnight at 4°C with 100 µl/well of UV-inactivated MV VACV<sub>WR</sub> (5 x 10<sup>7</sup> PFU/ml, and 51 µg/ml, prior to 1:25 dilution in PBS), as described (15, 57). Purified anti-D8 (LA5) and anti-H3 (#41) mAbs were used at 10 µg/ml. For linear peptide ELISA, biotinylated 20-mer peptides, overlapping by 10 residues and covering the D8 protein sequence were synthesized by A&A labs (San Diego, CA). Microtiter plates were coated with 100 µl of NeutrAvidin biotin-binding protein (1 µg/ml) diluted in
PBS overnight at 4°C (ThermoScientific Pierce, Rockford, IL). Then, coated plates were washed with washing buffer (PBS, pH 7.2, plus 0.5% Tween-20) and blocked with blocking buffer (PBS pH 7.2 plus 0.1% BSA plus 0.2% Tween-20) for 2 hours at room temperature (RT). Plates were incubated with 100 μl of biotinylated peptides (200 ng/ml) in blocking buffer for 90 min at RT. Plates were washed and incubated with purified mAb (LA5) at 10 μg/ml for 90 min at RT. Plates were washed, and the bound antibody was detected by adding a streptavidin-horseradish peroxidase (HRP) conjugated secondary antibody to mouse IgG (Invitrogen, CA) and incubated for 60 min at RT, followed by OPD substrate (Sigma-Aldrich).

**VACV MV neutralization.** VeroE6 cells were seeded at 1.5 x 10^5 cells/well into 24-well Costar plates (Corning Inc, Corning, NY) and used the following day (75-90% confluence). Two different VACV MV neutralization assays were used: (i) 1 hour no complement, and (ii) 1 hour plus 1% baby rabbit complement (Cedarlane laboratories, Burlington, Canada). Neutralization experiments were performed as described (34). The percentage of MV VACV neutralization was calculated and defined according to the formula: [(PNV – PNS) / PNV] x 100, where PNV was the average number of plaques by MV VACV WR alone or MV VACV WR + C'. PNS was the average number of plaques in the presence of each experiment sample.

**Surface plasmon resonance.** Surface plasmon resonance (SPR) studies were performed on a Biacore 3000 (GE Healthcare) to determine IgG2a LA5/VAVC D8 equilibrium binding affinity. Rabbit anti-mouse IgGs (Mouse antibody capture kit, GE Healthcare) were amine coupled to a CM5 chip using the amine coupling kit, according to the manufacturers suggestions (GE Healthcare). Mouse mAb LA5 was diluted in running buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% Tween 20) and ~ 300 response units (RU) were immobilized on the sensor chip. A mouse IgG1 mAb specific for a CD1d/glycolipid complex was used as a negative control for unspecific binding. Monomeric D8 Δ265 was isolated by SEC in SPR running buffer and passed over the LA5 immobilized sensor chip at 25°C with a flow rate of 30 μl/min using...
increasing concentrations of D8 (0.2-25.6 nM). Kinetic parameters were calculated using a simple Langmuir 1:1 model in the BIA evaluation software version 4.1.

**Fab digestion and purification.** Purified LA5 mAb (mouse IgG2a) at 1 mg/ml was incubated with 1% (w/w) activated papain for 3 h at 37°C in digestion buffer (50 mM Tris pH7.0). Papain was activated by incubating 20.80 µl papain solution (Sigma) with 100 µl 10 x papain buffer (1 M NaOAc pH5.5, 12 mM EDTA) and 100 µl cysteine (12.2 mg/ml) for 15 min at 37°C. The papain digestion was stopped by adding 20 mM Iodoacetamide (IAA). Two volumes of 3 M NaCl, 1.5 M Glycine pH8.9 were added to the digestion mix for subsequent protein A purification to remove undigested IgG and Fc. The protein A flow-through containing Fab was dialyzed against 50 mM NaOAC pH5.5 over night and purified to homogeneity by cation-exchange chromatography using MonoS (GE Healthcare).

**Fab/Ag complex preparation.** D8 and Fab were mixed together as homogeneous, monomeric species at equimolar ratio and at low concentration (<1 mg/ml). The D8-Fab complex was concentrated to a volume of 250µl using centrifugal filtration devices (Amicon Ultra 15, 10 kDa MW cut-off) and subjected to SEC purification (Superdex 200 GL10/300) to separate the D8-Fab complex from unbound D8 and/or Fab. Before crystallization, the nature of the complex was verified by non-reducing SDS-PAGE. Fractions corresponding to the complex were pooled and concentrated to ~10 mg/ml using centrifugal filtration devices.

**Crystallization and structure determination.** Initial crystallization experiments were carried out by sitting drop vapor diffusion in a 96-well format, using the Phoenix liquid handling robot (Art Robbins Instruments) and a panel of commercial sparse-matrix screens (PEG/Ion 1 and 2 from Hampton Research, Wizard 1 and 3 from Emerald Biosciences, JCSG+ Suite from QIAGEN, and JBScreen 6 from Jena BioScience). Quality diffracting crystals of LA5-Fab were manually grown at room temperature (RT) by mixing 0.5 µl of protein solution at 5.0 mg/ml with 0.5 µl of precipitant (10% (w/v) PEG 3000, 100 mM phosphate-citrate pH4.2, and 200 mM NaCl). D8 and D8-LA5-Fab crystals were grown in similar conditions (D8: 0.2 M sodium iodide,
20% PEG 3350; D8-LA5-Fab: 0.2 M potassium thiocyanate, 20% PEG 3350). Protein concentrations were 9.1 and 5.0 mg/ml, respectively. Crystals were flash-cooled at 100 K in mother liquor containing 20% glycerol. Diffraction data were collected at the Stanford Synchrotron Radiation Lightsource (SSRL) beamlines 7.1 and 11.1, and processed with the iMosflm software (3). Crystal structures were determined by molecular replacement (MR) with MOLREP as part of the CCP4 suite (12, 58), using a previously determined structure of the Fab of the anti-B5R (VACV) mAb B96 (unpublished) in combination with the homology model of D8 (based on PDB ID 3JXF) obtained with SWISS-MODEL (8) as search models.

Structures were refined by performing multiple runs of model building in Coot (21) followed by maximum likelihood restrained refinement with Refmac (39). At later refinement stages, ten cycles of translation/libration/screw-axis displacement (TLS) refinement were performed using the following five TLS domains assigned by the TLS motion determination server (TLSMD, (43)): variable heavy (1-115), constant heavy (116-225), variable light (1-109), constant light 1 (110-186), and constant light 2 (187-225). One TLS group was assigned for the entire D8 molecule.

At first, the D8-LA5 Fab complex structure was determined and the resulting coordinates for the Fab and D8 were subsequently used for structure determination of the separate D8 and LA5 Fab structures by MR. The quality of the models was examined with the program Molprobity (33). The D8-LA5 Fab complex structure was refined to 2.1 Å (R\textsubscript{cryst}=20.1%, R\textsubscript{free}=25.2%), the LA5 Fab structure to 1.6 Å (R\textsubscript{cryst}=19.8%, R\textsubscript{free}=22.9%) and the D8 structure to 1.42 Å (R\textsubscript{cryst}=17.4%, R\textsubscript{free}=19.0%) resolution. The coordinates and structure factors of D8, LA5-Fab, and D8/LA5-Fab complex have been deposited in the Protein Data Bank (www.rcsb.org) with accession numbers 4E9O, 4EBQ and 4ETQ, respectively. The nucleotide sequences of the LA5 antibody have been deposited in GenBank with accession numbers JQ815182 and JQ815183.

Data collection and refinement statistics for each of the three separate crystal structures are presented in Table S1. Figures were prepared using PyMOL (Schroedinger) and electrostatic
surfaces were obtained by converting the pdb file to a pqr file using the PARSE force field in the pdbtopqr server (19) (http://kryptonite.nbcr.net/pdb2pqr/), which creates an adaptive Poisson-Boltzmann solver (apbs) input file. The apbs plugin was used to generate the electrostatic surfaces in PyMOL.

D8/CS-4 docking. Docking experiments were performed with Autodock Vina using a D8 pdbqt formatted structure file along with chondroitin-4-sulfate (C4S) di- and tetrasaccharides as potential ligands, extracted from the CS/cathepsin K complex structure [pdb code 3C9E, (30)]. A first docking experiment over the full D8 surface with the C4S disaccharide determined the positively-charged crevice as the best scored binding site. A second docking experiment using the tetrasaccharide included the area around the crevice as for the docking grid.
RESULTS

Biochemical characterization of D8.

We prepared D8 Δ265 ectodomain consisting of residues 1-264. During expression of D8 Δ265, we noticed a propensity for higher molecular weight (MW) oligomers detected by size exclusion chromatography (SEC, Fig. 1A), indicating that D8 exists mostly as a tetramer. SEC carried out under non-reducing and reducing conditions indicated that D8 tetramer forms by the non-covalent association of two disulfide–linked dimers. A minority of D8 also exists as octamer and dimer. An equilibrium exists between the tetramer and the monomer species, while the disulfide linked dimer is hardly seen by SEC, supporting the notion of a rapid transition into the tetrameric state upon forming the disulfide-linked dimer. The equilibrium can be shifted toward the D8 monomer species under reducing conditions, while the tetrameric form dominates under non-reducing conditions (Fig. 1A). Peak fractions for the tetramer and monomer were analyzed by non-reducing SDS-PAGE, as well as by non-denaturing gel electrophoresis (Fig. 1B and C).

The SDS-PAGE illustrates the covalent nature of the dimer, since the protein migrates at approximately 70 kDa under non-reducing conditions and 35 kDa when reduced. The inter-molecular disulfide bond is likely formed through the only cysteine, C262, located near the C-terminal end of D8 Δ265, in the most membrane proximal region of the ectodomain. As D8 Δ265 only lacks the C-terminal ten residues of the full-length ectodomain (residues 265-274), the observed homotypic interaction of recombinant D8 Δ265 will likely also occur in the full-length D8 on the virus. We speculate that the oligomerization of D8 increases the viral binding avidity to CS, which would favor viral adhesion to cells expressing low levels of CS on the surface.

VACV D8 crystal structure.

To increase the likelihood of crystallization, we prepared the truncated construct D8 Δ262 (residues 1-261), in which the C262 necessary for disulfide-mediated dimerization was eliminated. This construct exists predominantly as a D8 monomer (Fig. S1). We then
determined the structure of the monomeric VACV D8 ectodomain to 1.42 Å resolution by X-ray crystallography (Table S1). At the center of D8 is a twisted ten-stranded central β-sheet, surrounded by four α-helices (Fig. 2A, C). The β-sheet is rather hydrophobic and three-fourth of its surface is shielded from the solvent by the surrounding loops and helices. The β-sheet platform runs equatorial through D8, separating the bottom half from the top half. The top half is characterized by a highly positively charged crevice in the center of the protein, lined by loops 4/β5 (Lys41 and Arg44), 7 (His80), 16 (His176) and 18 (Arg220 and Lys224) on one side, and loops 5 (Lys48) and 10/α1 (Lys98) at the other end (Fig. 2B, C). We have identified this positively charged crevice, by automated molecular docking routines (Autodock Vina (56)), as the potential binding site for CS, and it complements both charge and shape of the elongated tetrasaccharide used for docking (Fig. S2). The last thirty residues of the ectodomain are disordered in the structure, and would be expected to protrude out of the bottom of full length D8 and then connect to the viral membrane.

LA5 is a complement dependent neutralizing antibody that binds a conformational epitope. Recently, a number of mAbs targeting the D8 protein have been described (35). Importantly, these mAbs are derived from VACV-infected mice and represent the physiologic B cell response to the smallpox vaccine. One of these mAbs, LA5, is described here in more detail. LA5 is an IgG2a isotype (35), and binds recombinant D8 (Fig. 3A). LA5 failed to bind any of the 20mer peptides spanning the entire D8 amino acid sequence (Fig. 3B), suggesting that LA5 mAb targets a discontinuous conformational epitope on D8. We used Surface Plasmon Resonance (SPR) to determine the real-time binding kinetics of LA5 to recombinant D8. Recombinant, monomeric D8 binds to immobilized LA5 mAb with an equilibrium dissociation constant (Kd) of 0.18nM, indicative of high affinity binding (Fig. 3C). The binding is characterized
by a fast association phase \( (k_{on}=10.8 \times 10^5 \text{M}^{-1}\text{s}^{-1}) \), and a very slow dissociation \( (k_{off} = 1.92 \times 10^{-307} \text{s}^{-1}) \), resulting in a very stable non-covalent LA5-D8 complex.

To determine if the binding of LA5 mAb to D8 is functional, we performed neutralization assays where LA5 was pre-incubated with VACV\(_{WR MV}\) in the presence or absence of complement. Clone 41, an H3 specific antibody previously characterized to neutralize VACV infection, was used as a positive control (16). As shown in Fig. 3D, neither LA5 nor clone 41 alone is able to prevent VACV infection after a 1 hr pre-incubation of mAb plus VACV. However, upon addition of complement, 80-90% neutralization is observed for both mAbs. 1% complement alone has no effect on VACV (Fig. 3D). Our data suggest that complement is needed to increase the footprint of the anti-D8 Ig via C1q and C3, due to the presence of alternative VACV adhesion proteins. We conclude that LA5 binds native D8 protein on VACV virions and is able to neutralize VACV infection in the presence of complement.

**The structure of the D8 ectodomain in complex with the Fab LA5.**

To understand the structural basis of the specificity of the mAb LA5 in recognizing D8 we determined the crystal structure of the complex between LA5-Fab and D8 ectodomain to a resolution of 2.1 Å (Table S1; Fig. 4, Fig. S1). Two highly similar copies of the Fab/D8 complex (0.37 Å root-mean-square-deviation, RMSD) are present in the asymmetric unit (ASU) of the crystal and, therefore, the structure is reported for only one of the two complexes. The LA5-Fab binds to a discontinuous, conformational epitope located around the positively charged crevice of D8 (Fig. 2B, 4B). A detailed list of all atom contacts between Fab and D8 is provided in Table S2.

The binding interface between D8 and LA5 is very large, with a total of 2434 Å\(^2\) buried surface area (BSA). The heavy chain dominates the binding footprint, with a total of 1872 Å\(^2\) BSA, in contrast to the 562 Å\(^2\) BSA covered by the L chain (Fig. 4A). The LA5 D8 epitope is formed by twenty-three residues spanning a primary sequence distance of over 220 residues.
(Gln3, Leu5, Thr39, Gly40, Lys41, Arg44, Lys108, Asn145, Ile174, Asn175, His176, Ser177, Ser204, Leu205, Ile215, Glu217, Tyr219, Arg220, Asn221, Tyr223, Lys224, plus N218 and N226 if including water-mediated contacts), with seven residues sharing contacts with at least two complementarity-determining region (CDR) loops of LA5-mAb (Thr39, Lys41, Asn145, Asn175, His176, Glu217 and Arg220, Fig. 4B and C). Eighteen residues of LA5 contact the antigen D8, from 6 of the 6 CDRs of LA5 (L1: Ser31; L3: Trp91, Thr92, Tyr94; H1: Ser29, Asn31, Phe32, Trp34; H2: Met51, Asp53, Ser55, Glu56, Glu58, Arg60, Arg75; H3: Tyr102, Arg103, Asp105, Fig. 4C). While the L chain only forms 1 polar interaction (H-bond) and 15 VdW contacts, the H chain binds through an intricate network of polar interactions (16 hydrogen bonds and 11 salt bridges), with an additional 115 VdW interactions contributing to the vast majority of the Fab-Ag interactions (Fig. 5, Table S3). CDR H2 and H3 seem to contribute equally in terms of the number of interactions with D8, while eliciting some degree of difference in the nature of their interactions: CDR H3 electrostatic interactions with D8 are more frequent (7 vs. 4) and are very localized and backed by the hydrophobicity of the surrounding interface, while the H2/D8 interface is spread out and less hydrophobic (Fig. 5B and C). CDR H1 contributes the least to the binding interface, with only three H-bonds and 43 VdW interactions. Strikingly, a triad of negatively charged residues of CDR H2 (Asp53, Glu56 and Glu58) binds inside the positively charge crevice of D8. H2:Asp53 and Glu58 bind to Asn175 of D8, H2: E56 binds to Arg44 and H2: Glu58 binds to Lys108, indicating extensive electrostatic interaction between LA5H2 and D8 (Fig. 5B). Similarly, H3 residues Tyr102, Arg103 and Asp105 from several salt-bridges and H-bonds with D8 residues Glu217, Tyr219, Arg220 and Tyr223 (Fig. 5C). In conclusion, H2 and H3 together dominate and dictate the overall binding of LA5 to D8 and are responsible for the specificity of the antibody, as well as the overall stability of the complex. The light chain forms only one direct hydrogen bond with D8 (L3: Tyr94 with His176 of D8) (Fig. 5D). However, the most interesting feature about the light chain is the recruitment of highly ordered water molecules forming what we refer to as a water zipper (Fig. 5D; Table S4).
Four water molecules bridge D8-Gln3, Lys224 and Asn226 with L1-Ser31, L3-Thr92 and Tyr94. This water zipper is also largely conserved in the other complex of the unit cell.

**Predicted CS and LA5 binding site on D8 overlap**

Most noticeable, the antibody binds with CDR H1 and H2 above the central crevice of D8 that was also predicted to bind CS (Fig. 6A, B). In the CS docking experiment, one of the two 4'-sulfates of the CS disaccharide binds in an electropositively charged pocket, lined by D8 residue Lys108, while the other 4'-sulfate sits above D8 residue Arg220 (Fig. 6B). While Arg220 is in contact with both CDR H1 and H2 of LA5, D8 residue Lys108 is directly engaged by H2:Glu58 (Fig. 6B). The LA5 footprint on D8 reveals that the stretch along the positive crevice of D8, between Arg220 and Lys108, is a major binding site for LA5, as both CDR H2 and H3 together bind to several D8 residues in that area (Asn175, His176, Arg220, Fig.4). Therefore, the major energetic footprint of LA5 on D8 overlaps directly with the predicted binding site of CS and would likely outcompete CS binding to D8 through its high, sub-nanomolar binding affinity. As a result, LA5 likely prevents D8 binding to CS on host cells, thus leading to a reduced infectivity through the D8/CS axis. Loss of D8 activity, however, can be compensated for by VACV binding to HS through the adhesion molecules A27 and H3, as indicated by the lack of the antibody to neutralize in the absence of complement (Fig. 3D), and the normal infectivity of D8 deleted VACV in vitro.

**Structural changes upon antibody binding.**

The crystal structures of both the Fab LA5 and D8 in their unliganded form were also determined. These datasets give an opportunity to compare how the structure of the individual unbound proteins compare to their conformation in the Ab:Ag complex. We first investigated whether the epitope is rigid in D8, which would favor a lock-and-key mechanism for the binding of LA5-Fab. Therefore, we compared the discontinuous epitope in both complexes of the
asymmetric unit of the crystal (Fig. S3A; RMSD = 0.37 Å), as well as in D8 before and after LA5 Fab binding (Fig. S3B). When superimposing the unbound D8 epitope onto the one of the LA5-Fab bound complex, the RMSD is 0.56 Å over 186 atoms. Overall, the epitope forming residues superimpose well and no major structural changes occur in D8 upon antibody binding.

More noticeable structural changes, are observed for the antibody upon binding to D8, not withstanding that the backbone conformation of the CDR loops is preserved. A re-orientation of side chains is observed only within the CDR’s of the H chain, which correlates with the dominant role of the H chain in antigen binding. Notably, H1:Phe32 rotates about 45° to increase the hydrophobic and VdW interactions with D8, especially with residues Gly40, Lys41, Ser204, Ile215 and Glu217. H1:Trp34 is among the most dramatic changes as it rotates about 80° to form novel VdW interactions with D8 residues Ile174, Asn175, His176, as well as forming a highly energetic cation-pi interaction with Arg220 of D8. The third residue with a noticeable change in orientation is in CDR H3. Y102 tilts sideways to form two H bonds with E217 and Arg220 of D8 (Fig. 5C and 6C). Residues within CDR H2 are largely unchanged, except for Glu58 (Fig. 6C). Therefore, H2 has the optimal conformation to bind to the D8 crevice that also predicted binds CS, while residues of H3 have to re-orient for a perfect fit, especially Tyr102 and Arg103 that form very localized H-bonds and salt bridges with D8 (Glu217, Tyr219). In summary, although the Fab LA5 largely appears to bind with a lock and key mechanism, slight changes have to occur to allow antigen binding. Those changes result in novel interactions between D8 and LA5 (VdW, cation-pi and H bonds) and likely contribute to the affinity and stability of the antibody antigen complex.

Sequence alignment and structural comparison of D8 homologs.

A phylogenetic alignment shows that D8 is widely conserved throughout the poxviridae family (Fig. 7; Fig. S4). It utilizes primarily the CAH fold originally defined in the carbonic anhydrase protein family. We analyzed sequence conservation of some of the most relevant
orthologs of the Orthopoxvirus family. Compared side by side, D8 sequences are nearly identical in vaccinia, variola, monkeypox and other direct orthologs (Fig. S4; Fig. 7; Table S5).

Initially, all eighty-six orthopox D8 representatives were extracted from the Poxviridae Bioinformatics Resource center (PBR: http://www.poxvirus.org) and aligned against VACV Acam2000 D8, while fourteen representative sequences are show in the alignment (Fig. S4).

The variation spectrum is composed of 32 out of 304 residues in FL-D8, and 26 are located within the 234 residues of the D8 crystal structure. There are only substitutions and no deletions. Table S3 lists all possible variations, along with their occurrence ratios, calculated on the 86 available orthopox D8 sequences. As a first observation, Acam2000 D8 sequence is closest to the consensus sequence, as only five residues out of the 32 are under-represented over the 86 orthopox sequences, and this property may contribute to vaccinia's broad-spectrum infectivity. We also observe that variations occur mostly for surface residues, but residues that form the proposed CS binding crevice are mostly conserved. (Fig. S4, Fig. 7). This suggests that the central crevice is crucial for the function of the virus, which correlates with its predicted binding to CS. While mAb LA5 was raised against VACV WR D8, it likely exhibits cross-reactivity to most other orthopox viruses.

**Discussion**

Our study reports the structure of a major VACV surface protein, D8. We have identified a positively charged crevice running along the center of the CAH fold, which roughly corresponds to the so-called hydrophobic pocket that is adjacent to the catalytic site of CAH proteins. This hydrophobic pocket confers specificity for CAHs to bind alternative substrates, such as phenyl esters (24), and phenylthiocarbamides (PTC), which are CAH inhibitors, used for the treatment of glaucoma. (9). The size, shape and charge of the crevice make it a potential binding site for CS. Automated docking experiment, using the CS tetrasaccharide from the cathepsin K structure [pdb code 3C9E, (30)], positioned CS along the positively-charged crevice.
of D8, with similar interactions as observed for cathepsin K. Furthermore, we have shown that
the full-length ectodomain of D8 exists predominantly as a tetramer. This tetramerization may
be important for CS binding, as multivalent interactions generally increase affinity significantly.
Disulfide-linked dimerization of D8 also occurs, in agreement with a recent observation of the
Xiang lab. D8 antisera from VACV immunized mice contained antibodies that recognize a
protein with an apparent MW of 67 kDa, which disappeared upon the addition of a reducing
agent and thus was presumed to be the disulfide-linked D8 dimer (Figure S5)(35).

Our understanding of antibody-antigen recognition is still limited, particularly in the
context of physiological antibody responses to pathogen proteins that occur after infection or
immunization with live pathogens. This study provides insights into the recognition of D8 by the
neutralizing antibody LA5. The antibody binds across the whole width of D8 to a discontinuous
epitope with a footprint (2434 Å² BSA) that is at the far upper end of common antibody antigen
complexes (1400 < BSA < 2300 Å², (32)). In comparison, a neutralizing antibody targeting the
VACV protein L1 binds around the tip of L1, roughly burying 1560 Å². The LA5 binding footprint
is dominated by the H chain, while the L chain binds to D8 through recruitment of a water zipper
that complements the surface imperfection and distance between L chain and D8 and
conserved water molecules have been observed in a variety of antibody-antigen complexes
(10). A few conformational changes occur within the antibody upon binding to D8, while the D8
antigen is stabilized by its CAH fold and exhibits almost no conformational change. The
absence of major conformational changes indicates a lock-and-key binding interaction between
LA5 and D8 that is also supported by the fast association of the antibody as measured by SPR.

VACV neutralization with anti-D8 mAb is complement dependent, consistent with our
previous finding for the antigens H3 and B5 (4, 5, 34) and suggesting that the virion is able to
use two other attachment proteins (A27 and H3) to bind to host cell GAGs (14, 25, 31) even
when D8 is targeted by antibodies. This is further supported by studies showing that D8 deletion
strains replicate efficiently in tissue cultures (ref. (40) and (46), and Y. Xiang unpublished data).
indicating that targeting D8 might not be sufficient for direct neutralization in vitro. In vivo, where GAG expression is more limited than on cell lines, the four attachment proteins (D8, H3, A27, A26) may serve more specialized roles for facilitating VACV infection of cell types with differential GAG expression. Whether LA5 binding to D8 directly blocks the virion from interacting with CS, or whether the crevice coincidentally forms the binding site for LA5 due to potentially increased immunogenicity, is currently unknown. It is also conceivable that the high affinity interaction combined with the large and robust footprint of LA5 on the D8 surface is a prerequisite for recruiting complement resulting in effective neutralization. Conversely, tetramerization of D8 could potentially lead to an arrangement of D8 in which individual D8 molecules are covered by neutralizing anti-D8 mAbs, while other CS binding sites are not accessible to antibodies but can still bind CS. In that case, recruitment of complement to the D8-antibody complex would have an additional steric effect that further blocks access of D8 to CS. These possibilities can be examined in future studies.

A “holy grail” of infectious disease B cell biology research is the ability to predict antigen binding sites of antibodies. If such predictive power is possible, it will only be possible if substantially more Ab:Ag structure complexes are known for baseline algorithm development, using real pathogen antigens and antibody responses generated under physiological conditions. The determination of a D8: LA5 structure is a significant step for those efforts.

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References


**Figure Legends**

**Figure 1.** D8 Δ265 characterization. (A) Size exclusion chromatography (SEC) of D8 performed under non-reducing (-DTT) and reducing (+DTT) conditions. Molecular weight markers with sizes in KDa are shown as a reference (dotted line). (B) Corresponding 8-16% SDS-PAGEs to show migration of non-reduced and reduced D8 SEC samples (S) and peak fractions of the SEC (1, 2, 3, 4, and 5). (C) The corresponding non-denaturing PAGE (7.5%) confirms the nature of oligomeric states, with: A, aggregates; O, oligomers; T, tetramers; D, dimers and M, monomers.

**Figure 2.** D8 crystal structure and 2D-topology. (A) Cartoon representation of D8 in rainbow colors, from the N-terminal (blue) to the C-terminal extremity (red). The core domain of D8 is a ten-stranded twisted β-sheet, surrounded by four α-helices and various loops. (B) Electrostatic surface representation of D8. View as in (A) but 90° rotated toward the reader to highlight the deep and positively charged cavity (indicated by arrows). (C) 2D topology of D8 using the same color code as in (A). Loops are numbered in green.

**Figure 3.** LA5 IgG specificity and neutralization activity. (A) MAb LA5 binds recombinant D8 by ELISA. An anti-H3 mAb (#41) was used as a negative control. (B) Peptide ELISA using overlapping 20mer D8 peptides (peptides 1-35). Anti-D8 mAb LA5 showed reactivity to D8 but not to any of the overlapping D8 peptides, or recombinant H3 (H) or buffer only control (0). (C) Surface Plasmon Resonance sensorgram showing high affinity binding (K_D=0.18nM) of D8 to immobilized LA5 IgG. (D) In vitro VACV neutralization assays were done using anti-D8 mAb LA5 and anti-H3 mAb #41. Neutralization assays were done in the absence (left) or presence (middle) of complement (C'). VACV EV alone or plus complement were negative controls (right). Dashed lines in (D) indicate 50% neutralization. Data are representative of three or more independent experiments. Error bars indicate SEM in each group.

**Figure 4.** Structure of the D8/LA5-Fab complex. (A) Overall structure of the complex. The Fab is shown as a cartoon representation, with light chain (VL, CL) in green and heavy chain (VH, CH)
in orange, while the molecular surface of D8 is shown in grey. The major contribution to the
Ab/Ag interface comes from the heavy chain. Light chain buries only 562 Å² of surface, while the
heavy chain dominates binding with 1872 Å². Shape complementarity (Sc) measures the
generic surface complementarity of protein-protein interfaces (29). As Sc becomes
meaningless for surface areas that are too small, it has not been calculated for the light chain
(n/a). (B) LA5-Fab footprint on D8 surface, colored by individual CDR loops. The CDRs were
removed, but the D8 surface residues that are in direct contacts with individual CDR’s of LA5
are colored as follows; L1 – brown, L3 – yellow, H1 – green, H2 – cyan, H3 – blue, overlapping
contacts - purple. The arrow indicates the location of the positively charged crevice mentioned
in figure 2. (C) Summary of the residues involved in direct contacts. Bold residues may elicit
contacts with multiple CDRs and correspond to the purple ones in (B).

Figure 5. Details of D8/LA5 interactions. Interaction between heavy chain CDRs H1 (A), H2, (B)
and H3 (C) with D8 residues (grey). (D) The light chain water zipper. Five water molecules (red
spheres) mediate contacts between LA5 light chain and D8. One water molecule is in contact
with a glycerol molecule and D8 K224.

Figure 6. LA5 blocks D8 binding to CS and conformational changes upon antibody
binding. (A) Details of chondroitin-4-sulfate binding to D8 as determined by Autodock Vina. (B)
LA5 binds with H1 and H2 to the same area of D8. (C) Comparison of LA5 in the free form
(grey) and when bound to LA5 (rainbow color). CDR coloring according to Figure 4. Dashed
lines indicate VdW (yellow), salt bridges (red), H-bonds (brown), cation-pi (black), and water-
mediated (light blue) interactions occurring upon Ag to mAb binding and contacting D8 residues
are labeled.

Figure 7. D8 orthopox sequence variations. (A) VACV D8 structure with orthopox amino acid
variations colored in red. Only three residues of the variation spectrum are also part of the D8
epitope targeted by LA5-mAb. (B) Phylogenetic tree/dendrogram of CAH domain–containing
homologs, obtained using Seaview PhyML algorithm (22). CAH-like homologs include orthopox
and more distant MYXV and RFV representatives (m083L and gp083L), human carbonic
anhydrases (CAH) 4, 9, and 12, and extra-cytoplasmic anchoring domains of PTPRs. VACV:
vaccinia; VARV: variola; HSPV: horsepox virus; CMLV: camelpox virus; MPXV: monkeypox
virus; MYXV: myxoma virus; CPXV: cowpox virus; ECTV: ectromelia virus; RFV: rabbit fibroma
virus; RPXV: rabbitpox virus; RFV Rabbit fibroma virus; TATV: taterapox virus. For the orthopox
family, all 85 sequences of orthopox D8 were extracted from the Poxvirus Bioinformatics
Resource Center and 14 representatives, which cover the full mutation spectrum, were selected
for the final alignment in Fig. S4. Mutations are listed in Table S3 along with their occurrence
ratio.
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<th>LA5Fab CDR</th>
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<td>H3: Y102, R103, D105</td>
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