

# Protect, modify, deprotect (PMD): A strategy for creating vaccines to elicit antibodies targeting a specific epitope

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In creating vaccines against infectious agents, there is often a desire to direct an immune response toward a particular conformational epitope on an antigen. We present a method, called protect, modify, deprotect (PMD), to generate immunogenic proteins aimed to direct a vaccine-induced antibody (Ab) response toward an epitope defined by a specific monoclonal Ab (mAb). The mAb is used to protect the target epitope on the protein. Then the remaining exposed surfaces of the protein are modified to render them nonimmunogenic. Finally, the epitope is deprotected by removal of the mAb. The resultant protein is modified at surfaces other than the target epitope. We validate PMD using a well-characterized antigen, hen egg white lysozyme, then demonstrate the utility of PMD using influenza virus hemagglutinin (HA). We use an mAb to protect a highly conserved epitope on the stem domain of HA. Exposed surface amines are then modified with short polyethylene glycol chains. The resultant antigen shows markedly reduced binding to mAbs that target the head region of HA, while maintaining binding to mAbs at the epitope of interest. This antigenic preference is also observed with yeast cells displaying Ab fragments. Antisera from guinea pigs immunized with the PMD-modified HA show increased cross-reactivity with HAs from other influenza strains, compared with antisera obtained with unmodified HA trimers. PMD has the potential to direct an Ab response at high resolution and could be used in combination with other such strategies. There are many attractive targets for the application of PMD.

immunofocusing | antibodies | influenza | hemagglutinin | vaccine

Vaccines are among the most profound accomplishments of biomedical science and provide cost-effective protection against infectious disease. Many vaccines work by eliciting a neutralizing Ab response that prevents infection (1). However, for some infectious agents, it has not been possible to create an efficacious vaccine, and for others, the protection provided by vaccines is strain-specific.

In the case of influenza, the majority of Abs elicited by vaccination target the trimeric viral surface glycoprotein, hemagglutinin (HA) (2, 3). The 3D structure of HA consists of two regions, the head and the stem (4). Most of the HA-directed Ab response focuses on the head region, which is therefore considered immunodominant (2, 3). Amino acid residues on the surface of this immunodominant head region vary substantially among different strains and change continuously in a phenomenon referred to as antigenic drift (5). This variability, which leads to new circulating virus strains, coupled with the immunodominance of the head region, necessitates the production of new seasonal vaccines against influenza (5).

Strikingly, there is an epitope within the stem region of HA that is highly conserved among influenza strains and not subject to seasonal variation (6–12), likely because residues that form this epitope are critical for viral fusion mediated by HA (13, 14). Except in rare cases, there is no significant immune response toward the stem region (15). Nonetheless, Okuno et al. (16) isolated an mAb that targets this conserved epitope and demonstrated its broad neutralizing activity. Since the discovery of

this broadly neutralizing Ab (bnAb) 26 y ago (16), many other HA stem-binding bnAbs have been characterized (6–12). In addition, expression of such bnAbs protects mice from lethal challenges with a broad range of influenza subtypes (17). Taken together, these results suggest that if Abs targeting the conserved stem epitope could be elicited, it might be possible to create a universal flu vaccine (5, 18–20). Such a vaccine might provide cross-strain protection against all circulating and future pandemic strains (i.e., new strains transmitted from animals to humans, such as those that led to the 1918, 1957, 1968, and 2009 pandemics) of influenza (21).

Toward this goal, there has been substantial interest in directing a vaccine-induced Ab response toward the conserved stem region of HA. This would require avoiding the normal, immunodominant Ab response against the head. Strategies that aim to direct the immune system toward a particular region of a protein are referred to as “immunofocusing” (22).

Previous immunofocusing work, either against influenza or other infectious agents, has used a variety of approaches. The five most prominent examples are (i) epitope masking (23–28), (ii) epitope scaffolding (29–33), (iii) protein dissection (34–37), (iv) antigen resurfacing (38–40), and (v) cross-strain boosting (41–43). Epitope masking is a method that shields the immunodominant region of a protein, often using unnatural glycosylation sites, to discourage Ab formation. Epitope scaffolding aims to transplant a conformational epitope of interest onto a unique protein scaffold. Protein dissection removes undesirable or immunodominant epitopes from the native antigen. Antigen resurfacing uses site-directed mutagenesis to install less-immunogenic residues

## Significance

The discovery of broadly neutralizing Abs (bnAbs) against infectious agents such as influenza virus and HIV-1 has sparked interest in creating vaccines that focus an Ab response toward a particular epitope of a protein. These “immunofocusing” strategies have shown promise but are also burdened with inherent limitations. We introduce an immunofocusing method called protect, modify, deprotect (PMD) that uses a bnAb as a molecular stencil to create vaccine candidates that direct the immune response toward the epitope of the bnAb. PMD has the potential to provide epitope-specific immunofocusing, in a generalizable manner.

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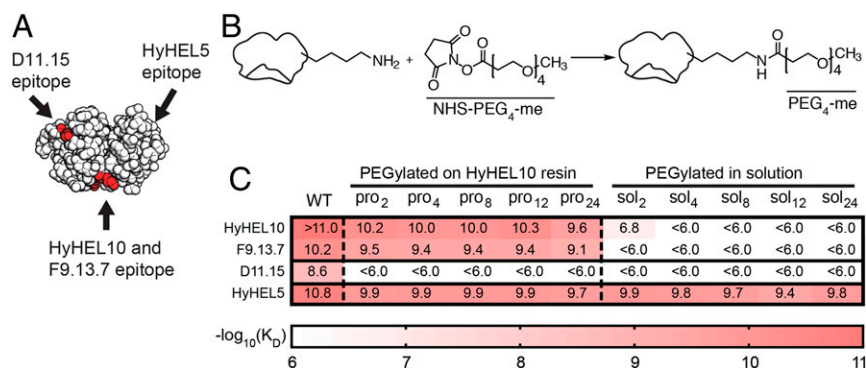
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**Fig. 2.** PMD with HEWL. (A) HEWL structure with the epitopes of D11.15, HyHEL10, F9.13.7, and HyHEL5 indicated by arrows and lysine residues in red. (B) NHS-ester reaction with a lysine residue is shown with NHS-PEG<sub>4</sub>-me. (C) Binding of mAbs to HEWL antigens PEGylated in solution (without PMD) or to PMD-generated HEWL antigens created with mAb HyHEL10 as the protecting Ab and using different PEG<sub>n</sub> lengths ( $n = 2, 4, 8, 12$ , or  $24$ ). Measurements were made using BLI. The  $-\log_{10}$  values for the dissociation constant ( $K_D$ ) are indicated. A heat map (scale at bottom) is overlaid.

measuring binding of the four mAbs to plates coated with the modified HEWL derivatives yield results that are fully consistent with these BLI results (*SI Appendix, Fig. S1C*).

We further analyzed the proteins PEGylated on and off of the HyHEL10 resin using SDS/PAGE followed by Ponceau S staining and Western blot analysis (*SI Appendix, Fig. S1B*). The results are generally consistent with those obtained by BLI (Fig. 2C); however, these analyses reveal a “laddering” phenomenon that is particularly prominent when longer PEGylation reagents are used (*SI Appendix, Fig. S1B*). Specifically, multiple discrete forms of PEGylated HEWL derivatives are observed, with molecular weight differences consistent with those expected for integral differences in the number of PEG<sub>n</sub> units. This suggests that PEGylation is incomplete in some cases. Likely candidate sites on HEWL that are incompletely PEGylated are K1 (the N-terminal residue), K96, and K97. Modification of the  $\epsilon$ -amino or  $\alpha$ -amino group of residue K1 may interfere with modification of the other group, and modification at either K96 or K97 may act to hinder modification of the adjacent residue. Therefore, longer PEG chains could be detrimental in efforts to fully PEGylate amines within unprotected epitopes.

Taken together, these results demonstrate that (i) protection with an mAb is required to retain the epitope of interest, since HyHEL10 did not bind to HEWL PEGylated in solution; (ii) PMD can selectively ablate binding of off-target Abs (in this case D11.15); (iii) use of longer PEGylation reagents can lead to incomplete modification; and (iv) the antigenicity of modified HEWLs can be predicted reasonably well with cocrystal structures, suggesting that holes can be predicted from 3D structural information.

**PMD with Influenza HA Using a Conserved Stem-Binding mAb (MEDI8852).** Given the ability to regulate the antigenicity of HEWL after PMD, we sought to design an immunogen that would elicit an Ab response to the conserved stem region of influenza HA by reducing the immunogenicity of the head. Such an immunogen should focus the immune system on the conserved HA stem, producing a more cross-reactive Ab response in immunized animals. We selected the stem-directed bnAb MEDI8852 as our protecting Ab (11).

To prepare a PMD-HA antigen, we started with HA $\Delta$ SA, an HA with a Y98F mutation to ablate sialic acid binding, which is based on A/New Caledonia/20/1999(H1N1), as described previously (54). We introduced a point mutation at the HA1/HA2 cleavage site to maintain the construct as HA0 (55) (*SI Appendix, Fig. S2A*) and added a foldon trimerization domain and purification tags at the C terminus (*Materials and Methods*). We refer to this construct as H1 WT. We used the crystal structure of a similar H1 HA [Protein Data Bank (PDB) ID code 4EDB] (56) to identify potential holes, here regions lacking surface lysine residues. We used deep mutational scanning data (57, 58) to identify residues within these predicted holes that can be replaced with lysine. In this way, nine lysine substitutions were made in the head region of H1 WT. We refer to this protein as H1+9 (*SI Appendix, Fig. S2B*).

To enable elution of H1+9 off of MEDI8852 resin following PMD while avoiding the irreversible conformational change that occurs with HA at low pH (13, 14), we used the cocrystal structure

of MEDI8852 with HA (PDB ID code 5JW4) (11) to install two point mutations, R52A and Y54A, in the MEDI8852 heavy chain. We refer to this mutated Ab as MEDI8852\*. These mutations lower the affinity of binding and facilitated elution of H1+9 off of a MEDI8852\* resin in 2 M KSCN at pH 7.4.

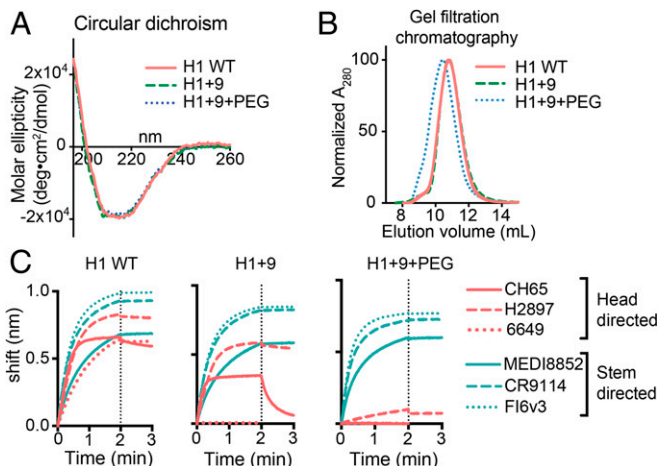
PMD was carried out as follows (*SI Appendix, Fig. S2C*). H1+9 was bound to MEDI8852\* resin and the complex was PEGylated with NHS-PEG<sub>4</sub>-me. The PEGylated H1+9 was eluted off the resin, producing a protein referred to as H1+9+PEG.

**H1+9+PEG Is a Properly Folded Antigen.** We sought to confirm that the structure of the protein was not perturbed by the PEG modifications. Thus, we compared the H1+9+PEG antigen to both H1 WT and H1+9 using gel electrophoresis, circular dichroism (CD) spectroscopy, gel filtration chromatography, and calorimetry. H1+9+PEG has a higher molecular weight than H1 and H1+9, as determined by SDS/PAGE. The molecular weight difference is consistent with that expected for PEGylation of approximately 20 amines on the surface of H1+9 (*SI Appendix, Fig. S2D*). Indistinguishable CD spectra for H1 WT, H1+9, and H1+9+PEG suggest that these proteins have the same folded structure (Fig. 3A). The gel filtration results for all three proteins are consistent with those expected for a trimer (Fig. 3B), with H1+9+PEG exhibiting a slightly earlier elution, consistent with an increased molecular weight due to PEGylation. Finally, calorimetry indicates that the proteins have a similar melting temperature (*SI Appendix, Fig. S2E*). Taken together, these results provide strong evidence that the HA antigen generated using PMD, H1+9+PEG, retains a native conformation.

To investigate whether it is necessary to protect the epitope during modification, we produced an antigen, denoted H1+9+sol, by PEGylating H1+9 in solution in the absence of MEDI8852\* (*SI Appendix, Fig. S3A*). H1+9+sol has a slightly higher molecular weight than H1+9+PEG as determined by SDS/PAGE analysis (*SI Appendix, Fig. S3B*), suggesting that additional PEGylation occurs in the absence of the mAb. The CD spectra of H1+9+sol and H1+9+PEG differ (*SI Appendix, Fig. S3C*). H1+9+sol melts at a lower temperature than H1+9+PEG, with an apparent pretransition, as determined by calorimetry (*SI Appendix, Fig. S3D*). Thus, H1+9+sol appears to exhibit a notable conformational change compared with H1+9+PEG. It remains to be determined if the main role of MEDI8852\* during PMD is to protect a lysine on the edge of MEDI8852's epitope from modification and/or to preserve the structure of the protein during modification.

**H1+9+PEG Maintains Stem Antigenicity and Decreases Head Antigenicity.** To determine whether the PMD protocol could maintain stem antigenicity and decrease head antigenicity, we used BLI to compare Ab binding to a set of six human mAbs, including three targeting the head and three targeting the stem (Fig. 3C). All six Abs bound to H1 WT (Fig. 3C, *Left*). Stem-directed Abs retained their binding after lysine substitution (H1+9) and after PEGylation (H1+9+PEG) (Fig. 3C, *Right*, blue). This demonstrates





**Fig. 3.** PMD with influenza HA. (A) CD spectroscopy comparing H1 WT, H1+9, and H1+9+PEG in 0.25× PBS. (B) Gel filtration analysis comparing H1 WT, H1+9, and H1+9+PEG FPLC in 1× PBS with a Superdex 200 Increase column. (C) Binding of anti-HA mAbs to H1 WT, H1+9, and H1+9+PEG as measured by BLI (head Abs shown in red; stem Abs, in blue). Association was monitored for 2 min (dotted lines), after which dissociation of the mAb was monitored for 1 min.

that the conformation of the HA stem is retained in the case of H1+9+PEG.

In contrast, head-directed Ab binding decreased after lysine substitutions (H1+9) and was further reduced after PEGylation (H1+9+PEG) (Fig. 3C, red). Notably, H1+9+PEG showed reduced but not ablated binding to the head Ab H2897, indicating the presence of an antigenic hole (as defined above) in the head of H1+9+PEG.

We envision two types of antigenic holes in PMD-created antigens. In one type, the hole is a result of incomplete PEGylation at a site. In the second type, PEGylation is complete, but the PEG moiety decreases the affinity of the probing Ab to a limited extent. To investigate these phenomena, we depleted our three protein samples for those that bind with high affinity to H2897 (*Materials and Methods*). Samples of H1 WT and H1+9 were depleted of all protein. In contrast, there was only partial depletion of H1+9+PEG (*SI Appendix, Fig. S4A*). Furthermore, the H1+9+PEG protein that was not depleted by our procedure showed decreased binding to H2897 with retention of binding to MED18852 (*SI Appendix, Fig. S4 B and C*). Thus, the H2897 hole apparent in Fig. 3C is likely a combination of the two causes outlined above.

Importantly, stem-directed Abs show decreased binding to H1+9+sol compared with H1+9+PEG (*SI Appendix, Fig. S3E*). It is likely that the PEGylation of a single lysine residue on the periphery of the MEDI8852 epitope or the conformational change that occurs when H1+9 is PEGylated in solution (see above) is responsible for this difference in binding. In either case, this result indicates that the PMD protocol is required to retain on-target antigenicity for MEDI8852.

**Yeast Expressing Antibody Fragments Show Preferential Stem Binding Toward H1+9+PEG.** Given that H1+9+PEG shows re-

**Binding toward H1+9+PEG.** Given that H1+9+PEG shows reduced binding of head Abs while retaining the binding of stem Abs, we sought to investigate antigenicity in a high-avidity situation (e.g., as would occur with a B cell population *in vivo*). A set of mAbs was expressed on the surface of yeast cells in the form of scFvs. It has been estimated that approximately 50,000 copies of scFv are expressed per cell using this protocol (59). Tetramers of H1 WT, H1+9, or H1+9+PEG, prepared by incubating biotinylated antigens with streptavidin, were used as bait in FACS experiments with four head-directed and six stem-directed yeast clones; representative FACS sorts are shown in [SI Appendix, Fig. S5A](#). Consistent with results obtained with isolated mAbs, the results with this high-avidity system indicate that all the stem Abs

that bind to H1 WT also bind to H1+9 and H1+9+PEG, while binding of the head Abs is significantly reduced (*SI Appendix, Fig. S5B*). In addition, the previously identified H2897 hole is apparent; approximately 4% of clones were antigen-positive.

Yeast display of scFvs also offers the possibility of generating libraries that can be used to detect holes in PMD antigens using FACS. As an initial experiment, we produced a mini-library of yeast expressing 22 different scFvs that bind to HAs of various subtypes. We pooled the 22 clones at an approximate equimolar ratio (Fig. 4, *Left*); performed FACS with H1 WT, H1+9, or H1+9+PEG tetramers; and sequenced the selected antigen-positive yeast. When the yeast library was sorted with H1 WT, there was no significant enrichment for either head or stem directed clones (Fig. 4). When sorted with H1+9, there was a slight enrichment for stem-directed clones; however, when the library was sorted with H1+9+PEG, there was a profound enrichment for stem-directed clones (Fig. 4, *Right*). These results show that H1+9+PEG is capable of enriching a polyclonal library for stem-directed clones and suggest that much larger libraries of scFv-displayed clones could be used to efficiently detect holes in PMD antigens in a high-throughput manner (see also ref. 60).

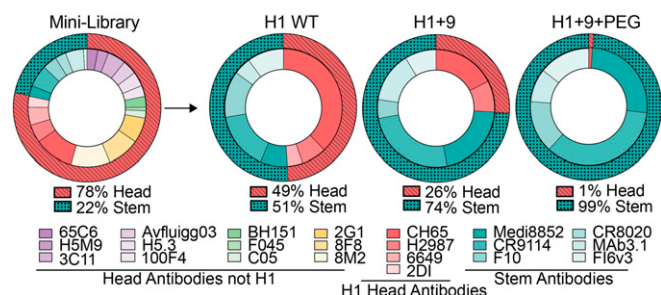
### H1+9+PEG Elicits More Cross-Reactive Serum Compared with H1 WT.

We evaluated the *in vivo* immunofocusing ability of PMD in guinea pigs. Given the foregoing results, we chose to compare H1 WT and H1+9+PEG as immunogens in Inject Alum adjuvant (Thermo Fisher Scientific) and boosted on day 20. This immunization experiment was done twice. The first experiment contained three animals in each group, and the second contained four animals in each group. A single animal (GP5) in the H1+9+PEG group produced a significantly weaker immune response (*SI Appendix*, Fig. S64) and thus was omitted from further data processing.

On average, antisera from animals immunized with H1+9+ PEG show slightly less binding to H1 WT, as determined by ELISA, compared with those immunized with H1 WT (*SI Appendix, Fig. S6D, Left*). This trend is also apparent at the individual animal level, as illustrated by serum EC25 titers, but the difference is not statistically significant (*Fig. 5, Left*). This result was expected, as PEGylation decreases the overall immunogenicity of proteins (46, 47, 61).

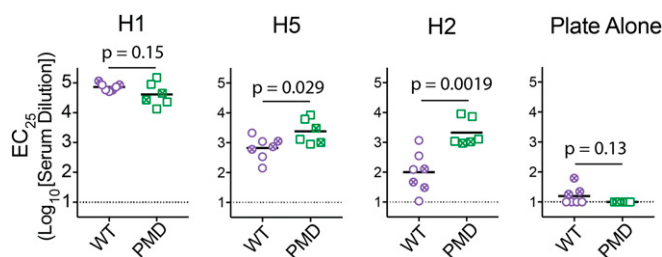
In contrast, ELISA results with the same H1+9+PEG antisera show more cross-strain binding to H5 HA compared with the H1 WT antisera (*SI Appendix, Fig. S6D*). The difference is even more pronounced with binding to H2 HA (*SI Appendix, Fig. S6D*). Comparing EC25 titers at the individual animal level indicates that these differences are significant (Fig. 5).

As a second method to evaluate antisera cross-reactivity, we used BLI with antisera from each group pooled in equal amounts from each animal. These BLI experiments confirmed that antisera from H1 WT immunized animals bind better to H1 WT than antisera from H1+9+PEG immunized animals but bind worse to H5 or H2 HA antigens (*SI Appendix, Fig. S6E*). Taken together,



**Fig. 4.** FACS of a mini-library of yeast-displayed scFvs using H1 WT, H1+9, or H1+9+PEG as the selection bait. The outer ring depicts the relative amounts of head- and stem-binding scFvs, and the inner ring represents the abundance of sequenced clones for the mini-library (*Left*) and antigen-selected clones (*Right*).





**Fig. 5.** Cross-reactive binding of antisera from guinea pigs immunized with H1 WT (purple circles;  $n = 7$ ) or H1+9+PEG (green squares;  $n = 6$ ). ELISA results (log EC<sub>25</sub>) for binding to H1, H2, or H5 HA trimers are depicted from two separate experiments, indicated as symbols with crosses and open symbols. Dotted lines indicate the limit of detection.  $P$  values were determined using the unpaired  $t$  test.

these results suggest that immunization with H1+9+PEG skews the Ab response toward the conserved stem epitope.

## Discussion

Our results demonstrate that PMD can serve as a generalizable and potentially high-resolution immunofocusing strategy that can be widely combined with other immunofocusing methods. With HEWL, we show that the PMD protocol keeps the epitope of interest intact while decreasing antigenicity elsewhere on the protein. With HA, we show that a PMD-generated antigen provides greatly reduced mAb binding at the head region while retaining robust binding to the stem region. With PMD-HA used as bait in FACS experiments, yeast clones expressing scFvs that bind to the stem region of HA were selectively enriched from a mini-library. Finally, when this PMD-HA antigen was used to immunize guinea pigs, the resultant antiserum was more cross-reactive to HAs from other influenza strains compared with animals immunized with unmodified HA. Although the *in vivo* derived effects are modest, taken together, our experiments demonstrate the viability of PMD for use in immunogen design.

Possible immediate steps to improve the efficacy of H1+9+PEG as an immunogen include (i) introducing additional lysine substitution(s) to eliminate the hole mapped by H2897 on the HA head, (ii) altering the PEG length or modifying reagent, and/or (iii) using other chemistries outside of NHS-esters (48). It will also be important to discover new holes that need to be eliminated with additional mAbs (e.g., with yeast-display scFv libraries). We imagine such improvements to be iterative, where new PMD candidates can be sequentially screened *in vitro* as outlined above before use *in vivo*. We also note that PMD vaccine candidates can be prioritized based on human B cell binding experiments (e.g., refs. 62 and 63).

Importantly, the PMD strategy is generalizable. It requires an antigen of interest and a mAb with an epitope against which to direct a vaccine-induced Ab response. 3D structural information is helpful but not absolutely required. Generating PMD antigens with a binding partner that is not an mAb is also conceivable, for example, using cell surface receptors such as CD4 for HIV-1 (64) or SR-B1 for HCV (65). Indeed, there are many attractive targets for the application of PMD.

We anticipate that another advantage of PMD is its potential to produce high-resolution epitope-focused vaccines. This is

because individual residues on an antigen either are or are not protected from chemical modification during PMD. Consequently, in theory, PMD could be used to create immunofocusing antigens at individual residue resolution. For example, it is conceivable that PMD could lead to vaccine candidates that avoid eliciting nonneutralizing Abs that bind to epitopes overlapping with those of neutralizing Abs (e.g., refs. 66–68).

Among the many possible applications of PMD, HIV-1 is particularly interesting to consider. The initial, immunodominant Ab responses to HIV-1 are strain-specific (69–71). While rare, bnAbs have been isolated from infected subjects and can be mapped to a few epitopes on HIV-1 Env (72, 73). The sequences of these bnAbs indicate that an extensive degree of somatic hypermutation generally occurs during years of viral and host coevolution (74, 75). PMD offers the possibility of creating immunogens to determine if it is possible to elicit a bnAb-like response in the absence of extensive somatic hypermutation, if other strain-specific Ab responses against HIV-1 are avoided.

Today, most vaccines are produced using methods developed many decades ago. Although in some cases these have had tremendous success, most notably the eradication of smallpox, they have failed to address some of greatest medical needs in the field of vaccinology, such as HIV-1 and influenza. Modern immunofocusing methods and the discovery of bnAbs have reignited the field to target such historically intractable diseases. Since PMD uses these bnAbs and can be used in combination with other immunofocusing strategies, we hope that it will aid in creating new vaccines.

## Materials and Methods

More detailed information is provided in [SI Appendix, Materials and Methods](#).

**Protein Expression and Purification.** We expressed all proteins except lysozyme (purchased from Alfa Aesar) in Expi293F cells and purified them using NiNTA (HAs) or protein A (Abs), followed by FPLC in some cases.

**PMD.** PMD was conducted by binding an antigen to an mAb resin (HyHEL10 for lysozyme or MEDI8852\* for H1+9), then incubating with an NHS-PEG-me reagent, and finally eluting the PMD-modified antigen off the resin using 100 mM glycine pH 1.5 for lysozyme or 2 M KSCN pH 7.4 for H1+9+PEG.

**BLI Measurements.** All BLI measurements were conducted using Octet Red96. All measurements were made in PBST+BSA buffer. A detailed experimental outline is provided in [SI Appendix, Materials and Methods](#).

**Yeast-Binding Experiments.** All yeast experiments were conducted in PBSTM buffer. Yeast were incubated with 12.5 nM of each tetrameric bait (H1 WT, H1+9, or H1+9+PEG) for 15 min, washed, stained again with an anti-c-Myc Ab (Milenyi Biotec) for 15 min, washed twice, and then flowed.

**Guinea Pig Immunizations.** Here 50  $\mu$ g of each immunogen (either H1 WT or H1+9+PEG) in 100  $\mu$ L was mixed 1:1 with alum adjuvant (Thermo Fisher Scientific) and immunized on day 0 and day 20. Serum was analyzed on day 30.

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# **Protect, Modify, Deprotect (PMD): A strategy for creating vaccines to elicit antibodies targeting a specific epitope**

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## **SUPPORTING INFORMATION APPENDIX**

### **Materials and Methods**

**HEWL:** HEWL was purchased from Alfa Aesar. Commercial sources are known to have a small amount of dimer<sup>1</sup>. PDB ID for HEWL is 1LYZ

**HEWL Monoclonal Antibody Cloning, Expression, and Purification:** Antibody sequences for HyHEL-10, HyHEL-5, D11.15, and F9.13.7 were taken from their sequences on the PBD (3HFM, 1YQV, 1JHL, 1FBI respectively). These protein sequences were codon optimized (using the IDT Codon Opt tool) for human protein expression. The heavy chain and light chain were then cloned into pFUSE-CHlg-HG1 vector and pFUSE2-CLlg-hK vector (InvivoGen), respectively, after the IL-2 signal sequence. These vectors, one containing the heavy chain and one containing the light chain of each antibody, were cotransfected into Expi293F cells (ThermoFisher). This was conducted by incubating 20 µg of each plasmid (40 µg total) in 1.5 mL opti-MEM (ThermoFisher) and mixing this with 1.5mL of opti-MEM containing 108 µl of expifectamine. This was incubated for 20-30 mins and added to 25.5mL of Expi-cells at 3 million cells/mL. These cells were then boosted 18-24 hours later with 150 µl of boost 1 and 1.5mL boost 2 (ThermoFisher). Cells were harvested by centrifugation at day 4 and the supernatant was diluted 1:1 into 1xPBS. This diluted supernatant was then flowed over a column containing protein A resin (ThermoFisher) at least two times or batch incubated for at least 1 hour, the resulting resin was washed with 10 column volumes of PBS and eluted with 100mM glycine pH 2.8 directly into 1/10<sup>th</sup> volume of 1M tris pH 8.0.

**Anti-HEWL HyHEL10 Column Resin Coupling:** To produce the HyHEL10-affinity resin, HyHEL10 was expressed in Expi cells at ~45mg/L. 8 mg of HyHel10 was coupled to an AminoLink® Plus Coupling Resin (ThermoFisher) using the pH 7.4 coupling protocol. Briefly, 8 mg of HyHEL10 in 3 mL of 1xPBS was added to 1 mL of AminoLink® Plus Coupling Resin pre-equilibrated in 1xPBS. To this mixture, 40 µl of 5M NaCNBH<sub>3</sub> in 1M NaOH was added, and the reaction was let to react overnight at 4°C, rotating. The resin was then washed with 1M Tris pH 8.0 and reactive sites were quenched with 3 mL of 1 M Tris pH 8.0 incubated with resin and 40 µl of NaCNBH<sub>3</sub> for 30 mins at room temperature. This resin was finally washed with 1 M NaCl to remove unconjugated protein. The theoretical binding capacity of the resin was ~700 µg of HEWL per reaction. The actual binding capacity was deduced to be ~500 µg indicating that some of the coupled antibody was in a nonproductive form.

**HEWL Resin PEGylation:** HEWL, 1mg/mL in PBS, was flowed over the HyHEL-10 affinity resin to saturation. This was subsequently washed 3x with 1xPBS. The resin was then incubated with NHS-PEG<sub>n</sub>-Methyl (Quanta Biodesign) at 3 mM such that there were 10 molar equivalents

of NHS ester per theoretical exposed (free) amine. This was incubated for 2 hours at room temperature rotating. This was then let to flow out of the resin and another incubation with NHS-PEG<sub>n</sub>-Methyl at 3mM was added to the resin for 2 hours rotating. NHS-PEG<sub>n</sub>-Methyl could be any of the following n=2,4,8,12,24 PEG lengths. The resin was then washed 2x with 100 mM Tris pH 8 to quench any unreacted NHS esters and eluted three times with pH 1.5 glycine directly into 1/5<sup>th</sup> volume equivalent of 1 M Tris pH 8 to neutralize the pH. pH was tested and further adjusted with 1 M Tris pH 8 if needed.

**HEWL Solution PEGylation:** HEWL was PEGylated in solution in the absence of the protecting antibody. This was done by taking a known amount of HEWL and adding 10 molar equivalents of NHS-PEG<sub>n</sub>-Methyl per theoretical exposed (free) amine (to a final concentration of 3mM). This reaction was left to react for 2 hours at room temperature and then an additional 10 molar equivalents of NHS-PEG<sub>n</sub>-Methyl was added and left to react for 2 hours. This reaction was then purified away from any organic solvent (the NHS ester is dissolved in DMSO or DMF) using a Zeba<sup>TM</sup> Spin Desalting Column 7k MWCO, as per the manufacturer's recommendation, pre-equilibrated in 1x PBS.

**Western Blots – HEWL Analysis:** After SDS-PAGE analysis, Western blots were conducted by transferring the SDS-PAGE minigel (BioRad) using the mixed molecular weight setting on a Transblot<sup>®</sup> Turbo<sup>TM</sup> (BioRad). The western blots were first Ponceau stained. The nitrocellulose blot is submerged in Ponceau S Solution (Sigma Aldrich) for 3 mins rocking, the resulting blot is then washed in deionized water until protein bands are apparent. The Ponceau stain is then washed off with continuous rounds of PBST until all the stain is removed. The blots are then blocked using 2.5% milk for 30 mins at room temperature, or overnight at 4°C. To this 3 µl of 1 mg/mL HyHEL-5, HyHEL-10, D11.15, or F9.13.7 was added and left to incubate for at least 1h at room temperature rocking. This was washed 3x with 1xPBST and goat-antihuman horseradish peroxidase (HRP) secondary (GenScript) antibody was added as per the manufacturer's recommendation and left to rock for at least 1h at room temperature. This was washed 3x with 1xPBST and developed using Pierce ECL Western Blotting Substrate or Pierce ECL Plus Western Blotting Substrate (ThermoFisher). Western blots were read on a GE AI600 RGB Gel Imaging System. If the western blot was to be screened against multiple of these antibodies, then the blot was stripped after imaging. This was done by first washing the blot 2x in deionized water and then 7mL of 1x Restore<sup>TM</sup> Western Blot Stripping Buffer (ThermoFisher) was added and let incubate for 7mins rocking at room temperature. This blot was then washed 2x with PBST and 2.5% milk was added for 10mins before another primary antibody was added. The same procedure was then followed for further development of the western blot.

**Biolayer interferometry (Octet) Binding Experiments – HEWL Analysis:** All reactions were run in PBS with 0.1% BSA and 0.05% Tween 20 (PBST BSA). Monoclonal antibodies expressed as above were loaded onto the (AHC) anti-IgG Fc Capture Biosensors at 100nM and the load threshold was set at 1nm. After loading, the tips were washed and then were associated, immersed, in 100nM of either HEWL WT, HEWL PEGylated on resin with PEG<sub>n</sub>, or HEWL PEGylated in solution with PEG<sub>n</sub>. This step was left to run for 90sec and then the biosensors were moved to PBST BSA to dissociate for 10mins. The resulting tips were then regenerated in pH 1.5 glycine and neutralized in PBST BSA 3 times before reloading monoclonal



antibodies (this regeneration step was also conducted on the tips prior to the experiment). All samples in all experiments were baseline subtracted to a well that loaded the tip with antibody, but did not go into sample, as a control for any buffer trends within the samples. The resulting binding curves were fit in GraphPad Prism to determine the  $K_D$  of the interactions.

**HEWL PEGylated ELISAs:** HEWL either WT or PEGylated on resin or PEGylated in solution was plated at 50uL in each well on a microtiter plate at 1ug/mL in 50mM sodium bicarbonate pH 8.75. This was left to incubate overnight at 4°C and then washed 3x with PBST using an ELx 405 Bio-Tex plate washer and blocked with 300uL of PBST + .5% BSA overnight at 4°C. The block was removed and serial dilution of monoclonal antibodies (described above) were added, starting at 100nM and undergoing 10-fold serial dilutions. These were left to incubate for 1 hour at room temperature and then washed 3x with PBST. Goat anti-human HRP (abcam ab7153) was added at a 1:50,000 dilution in PBST. This was left to incubate at room temperature for 1 hour and then washed 6x with PBST. Finally, the plate was developed using 50 µL of 1-Step™ Turbo-TMB-ELISA Substrate Solution (ThermoFisher) per well. Finally, the plates were quenched with 50 µL of 2M H<sub>2</sub>SO<sub>4</sub> to each well. Plates were read at 450 nm and normalized for path length using a BioTek Synergy™ HT Microplate Reader. Lastly the samples were baseline subtracted by subtracting the average of wells containing only secondary antibody.

**HA Protein Cloning:** Hemagglutinin ectodomains constructs of H1 (AHJ09883.1) was as previously described<sup>2</sup> with an R343G mutation to discourage cleavage and cloned into the pADD2 backbone as previously described<sup>3</sup>. We replaced the native leader sequence of the H1 construct with an IL-2 leader sequence at the N-terminus. To the C terminus of the ectodomain, a foldon domain, an AviTag™, and hexa-HIS tag (in this order) were added to enable purification and biotinylation. A Y98F mutation was made to ablate sialic acid binding, and permit easier protein purification as previously described by Whittle, J. R. et al., *J Virol* 88:4047–4057 (2014). The foldon domain constitutes the C-terminal 30 amino acid residues of the trimeric protein fibrin from bacteriophage T4, and it is added to the HA ectodomains to cause the expressed proteins to trimerize. The AviTag™ is a 15 amino acid peptide tag that is site specifically biotinylated (at a lysine residue) by E. coli biotin ligase (BirA). The hexa-HIS tag facilitates purification using nickel affinity chromatography. Constructs were also produced where the foldon-avi tag were replaced by a linker region (GGGGTGGGGTG) and an IZ tag (RMKQIEDKIEEILSKIYHIENEIARIKKLIGER) to facilitate trimerization. These constructs contained a 8xHis tag to facilitate purification. Identical constructs (only containing the Y98F mutation) were made with containing their native signal peptide for both an H2 HA protein — A/Japan/305+/1957 (H2N2) (Xu R, McBride R, Paulson JC, Basler CF, Wilson IA (2010) *J Virol* 84(4):1715–1721.) and an H5 HA protein — a/Viet Nam/1194/2004 (H5N1) (Govorkova EA, et al. (2005) *J Virol* 79(4):2191 LP-2198.) with a linker-IZ-his tag construct (H1 IZ, H2 IZ, and H5 IZ).

**HA Antibody Cloning:** Antibody sequences were cloned into the CMV/R plasmid backbone for expression under a CMV promoter. The antibodies variable HC and variable LC were cloned between the CMV promoter and the bGH poly(A) signal sequence of the CMV/R plasmid to facilitate improved protein expression. This vector also contained the

HVM06\_Mouse (P01750) Ig heavy chain V region 102 signal peptide to allow for protein secretion and purification from the supernatant. The antibody sequences were either taken from the protein sequence (obtained from crystal structures in the RCSB Protein Databank) and codon optimized (IDT CodonOpt tool) or from the reported NCBI Accession number as provided in the Table below. All constructs were designed such that there was a 12-15 base pair overlap with the open (cut) CMV/R vector. Cloning was performed using the In-Fusion® HD Cloning Kit master mix (Clontech). A mutated Medi8852 antibody was generated that had decreased binding affinity towards its HA stem epitope and allow for elution off of an affinity resin made of this modified antibody. Two mutations were identified by examining the crystal structure that should decrease the binding affinity upon alanine mutation. Both residues were within the HC of the known Medi8852 antibody. Residues 52 and 54 were both mutated to alanine residues (HC R52A, HC Y54A). The constructs were cloned into the CMV/R plasmid backbone using the In-Fusion Cloning Kit (Clontech). Cloning of scFv constructs is described below.

**Table S1.** Monoclonal antibody sequences.

Antibody Name	Crystal Structure	Accession Number HC	Accession Number LC	Target	Produced as MAb or scFv on yeast
Medi8852	5JW4	KX398437.1	KX398446.1	HA Stem	Both
65C6	5DUM	JF274050.1	JF274051.1	H5 Head	ScFv on yeast
H5M9	4MHH	KF500000.1	KF499999.1	H5 Head	ScFv on yeast
3C11	NA	JF274048.1	JF274049.1	H5 Head	ScFv on yeast
AvifluIgG03	5DUP	NA	NA	H5 Head	ScFv on yeast
100F4	5DUR	JF274052.1	JF274053.1	H5 Head	ScFv on yeast
H5.3	4XNM	NA	NA	H5 Head	ScFv on yeast
BH151	1EO8	AJ251890.1	AJ251891.1	H3 Head	ScFv on yeast
F045	4O58	AB649270.1	AB649271.1	H3 Head	ScFv on yeast
CO5	4FP8	JX206996.1	JX206997.1	H3 Head	ScFv on yeast
2G1	4HG4	JN130392.1	JN130393.1	H2 Head	ScFv on yeast
8F8	4HF5	JN130388.1	JN130389.1	H2 Head	ScFv on yeast



8M2	4HFU	JN130390.1	JN130391.1	H2 Head	ScFv on yeast
CH65	5UGY	NA	NA	H1 Head	Both
H2987	5UG0	NA	NA	H1 Head	Both
6649	5W6G	NA	NA	H1 Head	Both
2DI	3LZF	EU825949.1	EU825950.1	H1 Head	ScFv on yeast
CR9114	4FQI	JX213639.1	JX213640.1	HA Stem	Both
F10	3FKU	NA	NA	HA Stem	ScFv on yeast
CR8020	3SDY	NA	NA	HA Stem	ScFv on yeast
MAb 3.1	4PY8	NA	NA	HA Stem	ScFv on yeast
FI6v3	3ZTN	NA	NA	HA Stem	Both

**Protein Expression and Purification:** All proteins were produced in Expi HEK293 cells as per the manufacturer's recommendation. Briefly, the cells were maintained in Expi293 expression media (ThermoFisher) and passaged every 3-4 days. The day before transfection Expi HEK293 cells were resuspended to a density of 3 million cells/mL and left to grow overnight. The following day the cells were diluted back to 3 million cells/mL and transfected. These vectors, one containing the heavy chain and one containing the light chain of each antibody, were cotransfected. This was conducted by incubating 20 µg of each plasmid (40 µg total) in 1.5 mL opti-MEM (ThermoFisher) and mixing this with 1.5 mL of opti-MEM containing 108 µl of expifectamine. This was incubated for 20-30 mins and added to 25.5 mL of Expi-cells at 3 million cells/mL. These cells were then boosted 18-24 hours later with 150 µl of boost 1 and 1.5 mL of boost 2 (ThermoFisher). Cells were harvested by centrifugation at day 4 and the supernatant was diluted 1:1 into 1xPBS. This diluted supernatant was then flowed over a column containing protein A resin (ThermoFisher) at least two times, washed with 10 column volumes of PBS and eluted with 100 mM glycine pH 2.8 directly into 1/10th volume of 1 M Tris pH 8.0. Hemagglutinin constructs were transfected at 30 µg plasmid DNA in 1.5 mL Opti-MEM and 80 µl of expifectamine per 25.5 mL of Expi cells. Cells were harvested by centrifugation and supernatant from cells expressing HA was diluted 1:1 in 1xPBS and purified using nickel affinity chromatography using Ni-NTA agarose (ThermoFisher). All ratios were scaled up when required.

**Lysine Substitution Mutations into H1 HA:** To determine amino acid residues that could potentially be mutated into lysine residues, available data on BLAST was used together with data from a previously described single point mutation library (57, 58). A clone was generated containing nine additional lysine at sites L60K, N71K, T146K, N155K, R162K, N176K, V182K, G202K, R205K of the H1 HA protein. The construct was cloned into the pADD2 vector using the

In-Fusion® Cloning Kit (Clontech). The final clone was sequence verified (Sequetech, Mountain View, CA). The protein expressed from this clone is referred to as “H1 +9.”

Protect, Modify, Deprotect – Anti-H1 Medi8852 Column Resin Coupling: Four milligrams of Medi8852 with 2 point mutations in the heavy chain (R52A, Y54A) was coupled to AminoLink® Plus Coupling Resin (ThermoFisher) as per the manufacturer’s recommendation (pH 7.4 protocol). Briefly, 4 mg of Medi8852 R52A Y54A in 2 mL, was incubated with 500 µl of AminoLink® Plus Coupling Resin (ThermoFisher) that had previously been washed with 1xPBS pH 7.4. To this mixture 40 µl of 5M NaCNBH<sub>3</sub> was added and left to react overnight, rotating at 4°C. The resin was subsequently washed with 1xPBS and quenched with 2 mL of 1 M Tris pH 8 with 40 µl of 5M NaCNBH<sub>3</sub>. Finally, the resin was washed with 1xPBS and at least 10 resin bed volumes of 1 M NaCl until no protein was detected in the flow through. This resin was stored in 1xPBS with NaN<sub>3</sub> (0.02%). All ratios were scaled up when required.

Protect, Modify, Deprotect – HA Head Protection: H1+9 (1mg/mL in 1xPBS) was batch incubated with the Medi8852 R52A, Y54A resin for 15 min rotating. This was washed 2x with 1xPBS and then 1xPBS with 3 mM NHS-PEG4-Methyl was added such that there were 5 molar equivalents per theoretical exposed (free) lysine residues. This was incubated for 45 min rotating, let drain, and then a second equivalent of this was added for another 45 min rotating. Finally, the resin was washed 1x with 100 mM Tris pH 8 to quench any unreacted NHS ester and wash out hydrolyzed NHS ester. The modified H1+9 was eluted in 2 M KSCN dissolved in 1xPBS at pH 7.4, directly into 3 equivalents of 1xPBS. This was immediately put into overnight dialysis in 1xPBS. The subsequent solution was concentrated. The resulting antigen where the H1+9 was PEGylated with NHS-PEG<sub>4</sub>-Me on a resin of Medi8852 HC R52A, HC Y54A resin is referred to as H1+9+PEG. If the reaction was done without resin binding (PEGylated directly in solution) the same molar equivalents of NHS-PEG<sub>4</sub>-Me per lysine residue were added directly to a solution of H1+9 in 1xPBS. The resulting protein was buffer exchanged using overnight dialysis.

BirA Biotinylation: BirA, expressed in E. coli, was used to biotinylate purified hemagglutinin derivatives. 1mL of the HA at 1mg/mL in PBS was mixed with 100 µl of 10X reaction buffer (0.5M Bicine pH 8.3, 500uM biotin final concentrations 50mM, pH 8.3, 50uM respectively) and 100 µl of 100 mM ATP stored separately (final concentration 10mM). 2.5 µl of 1mg/mL BirA stock solution was added and the reaction was incubated at 37°C for 1 hour. A PD-10 column (SEPHADEX) was used to buffer exchange the HA derivatives. This reaction was conducted before PEGylation of H1+9 if the resultant protein was to be used in a biotinylated form.

HA Deglycosylation: PNGase F (NEB) was used to deglycosylate HA constructs for SDS-PAGE analysis as per the manufacturer’s recommendation. Briefly, 20µg of HA in 9µL H<sub>2</sub>O was added to 1µL of Glycoprotein Denaturing Buffer (NEB). This was boiled at 100°C for 10 mins 2µL of NP-40 and Glycobuffer 2 was added with 1µL PNGase F and the final reaction volume was made to 20µL and left to incubate at 37°C for 1 hour. The resulting protein was used in SDS-PAGE analysis.

CD Spectroscopy: All CD samples were prepared by first dialyzing into .25x PBS overnight with 1x change of dialysis buffer. The resulting buffer in the dialysis container was used as the buffer blank. The samples concentration was measured using Nanodrop 2000 (ThermoFisher) blanking with the dialysis buffer. CD spectra were determined using Jasco J-815 CD Spectrometer sampling every .5nM between 260nM and 180nM and 5 accumulations were collected and averaged. Finally, the samples were buffer subtracted using the dialysis buffer blank run under the same conditions. Data is reported until the voltage of the buffer sample reached 400V.

Gel filtration chromatography (FPLC): Samples for the second immunization were FPLC purified using an AKTA pure FPLC with a Superdex 200 Increase gel filtration column (S200). 1mL of sample (~1-3mg) was injected using a 2mL loop and run over the S200 which had been preequilibrated in degassed 1xPBS prior to use. Samples A280 was exported and normalized using Prism Graphpad.

Calorimetry: Thermal melts were determined using the Prometheus NT.48 made by Nanotemper. Samples, first dialyzed in .25x PBS at ~.1mg/mL, were loaded into Prometheus NT.Plex nanoDSF Grade High Sensitivity Capillary Chips and the laser intensity was set to 100%. Samples were let to melt using the standard melt program and the first derivative was plotted and normalized using Prism GraphPad.

H1 Biolayer interferometry (Octet) Binding Experiments: All reactions were run on an Octet Red 96 and samples were run in PBS with 0.1% BSA and 0.05% Tween 20. To assess Head vs Stem H1 antibody binding to immunogens, binding was determined by using streptavidin (SA) biosensors (ForteBio) loaded for 5 min with 18 nM biotinylated antigens and the load threshold was set to .8nm. Tips were then associated in 100 nM of each antibody and left to dissociate in the original buffer wells for 60 seconds. These curves were baseline corrected and exported and plotted on GraphPad (Prism 7). For comparisons between the H1+9+PEG and H1+9+sol, the proteins were loaded onto HIS1K octet biosensors at 50nM and the load threshold was set to .22nm and then washed 1x before being associated with 100nM of either of the 4 stem directed antibodies. For comparison between H2897 depleted and un-depleted H1+9+PEG SA biosensors were used and a threshold was set to .7nm. The threshold was used as the proteins were at different concentrations and therefore, this was threshold was used to normalize the amount. After loading, association in H2897 (100nM) or medi8852 (15nM) was done for 4mins and then dissociation for 1min.

H2897 Depletion of H1+9+PEG: 50μL of 1.5μM H1 WT, H1+9, or H1+9+PEG were added to either 50μL of 1xPBS or 50μL of 10μM H2897 and left to incubate for 1 hour at room temperature. The resulting solutions were incubated with 50μL of protein A resin (ThermoFisher) for 4 hours at 4°C. Resin was isolated by spinning through an ultrafree-MC centrifugal filter (Milipore Sigma) and the resulting solution was incubated again with another 50μL of protein A resin for 1 hour at 4°C. The final solutions were isolated by spinning through another ultrafree-MC centrifugal filter to remove the resin.

Western Blots – H2897 Depletion Analysis: After SDS-PAGE analysis, Western blots were conducted by transferring the SDS-PAGE minigel (BioRad) using the mixed molecular weight setting on a Transblot<sup>®</sup> Turbo<sup>™</sup> (BioRad). The blot was then blocked using 15 mL of 3% BSA for 30 mins at room temperature. To this 3  $\mu$ L of streptavidin HRP (BD ELISPOT) was added and left to incubate for at least 1h at room temperature rocking. This was washed 3x with 1xPBST and developed using Pierce ECL Western Blotting Substrate or Pierce ECL Plus Western Blotting Substrate (ThermoFisher). Western blots were read on a GE AI600 RGB Gel Imaging System.

Yeast Cloning: Yeast clones were produced by cloning the scFv of the antibodies set forth in the above table into a pPNL6 backbone. ScFvs were designed using the yol tag as the linker between the HC and the LC of the antibodies. All scFvs were designed in the order HC-yol tag-LC. Clones were sequence confirmed and transformed as previously described. Briefly, yeast were grown on a YPAD plate and then a single colony was inoculated in 5 mL of YPAD overnight shaking at 30°C. 200  $\mu$ L of YPAD culture were harvested per clone and pelleted. Carrier DNA (salmon sperm DNA (Sigma)) was boiled for 5 min, and the aliquots were stored frozen. 24  $\mu$ L PEG 3350 (50% w/v), 3.6  $\mu$ L lithium acetate (1 M), 5  $\mu$ L boiled carrier DNA (2 mg/mL), plasmid DNA (0.01-0.1  $\mu$ g), and water (up to 36  $\mu$ L) was added to each YPAD pellet. This was incubated at 42°C for 2 hours. Cells were harvested by centrifugation and resuspended in 100  $\mu$ L of water. 10  $\mu$ L of each clone was plated on selective agar plates lacking tryptophan and grown at 30°C for 3 days until colonies were visible. Yeast were picked and grown in SD-CAA media overnight (30°C shaking) and then induced by a 1:100 dilution into SG-CAA media and grown at 20°C shaking for 2 days.

Yeast Binding Experiments – Individual Clone Binding: Following induction in SG-CAA shaking for 2 days at 20°C, yeast clones, each expressing a different scFv on their surface, were separately incubated for 15 mins with 12.5 nM tetrameric bait in 50  $\mu$ L PBSM. Tetrameric baits were pre-formed with 50 nM biotinylated antigens and 12.5 nM streptavidin 647 (Jackson ImmunoResearch) for each of the respective antigens. Cells were then washed 1x with PBSM and then incubated with 1  $\mu$ L of anti-c-myc FITC (Miltenyi) in 50  $\mu$ L PBSM for 15 mins. Samples were then washed 2x with PBSM and then resuspended in 50  $\mu$ L of PBSM. These samples were flowed (Accuri C6 flow cytometer) and the percent antigen positive was determined as the ratio of antigen positive cells divided by all cells expressing scFv (c-myc positive). Gates were set such that ~.1% of yeast were antigen positive in the streptavidin alone control.

Yeast Binding Experiments – Polyclonal Sorts: Yeast clones were pooled based on their concentration such that a near equimolar ratio of each clone was added to the 'library'. This library was made independently twice, producing a biological duplicate of the yeast library. Both biological duplicate libraries were treated as above, such that an aliquot of the entire yeast library was incubated with 12.5 nM of each of the different tetrameric baits, produced as above. As such, there were 6 independent yeast incubations, one per biological duplicate, for each of the 3 antigens, H1 WT, H1+9, H1+9+PEG. These were incubated for 15 mins with the tetrameric baits, after which the yeast libraries were washed once with 1xPBSM and incubated with 1  $\mu$ L of anti-c-myc FITC (Miltenyi) per 50  $\mu$ L of yeast in PBSM. Samples were then washed 1x



with PBSM and then resuspended in PBSM. These libraries were then sorted on a FACS machine (SH800S Sony). The samples were gated such that all antigen positive cells were collected (gates set such that  $\sim 1\%$  streptavidin alone controls fell within the gate). 100,000 cells were sorted in each case. These sorted libraries were grown for 2 days at  $30^{\circ}\text{C}$  shaking in SD CAA media and then 100  $\mu\text{L}$  of the cultures were minipreped (Zymo Research) and transformed into STELLAR Competent Cells (Clontech) and plated on carbenicillin LB agar plates (as per pPNL6's resistance marker). *E. coli* cells that grow should, theoretically, contain only a single sequence from each of the yeast that were sorted above. *E. coli* colonies from each sort (a total of 101 (full library), 188 (WT), 115 (H1+9), and 97 (H1+9+PEG) were sequenced) were sent for sequencing (MClabs, South San Francisco). The sequences were then analyzed by sequence alignment using SnapGene software.

**H1 WT and H1+9+PEG Immunizations:** Groups of guinea pigs (3+4 each) were immunized, each group immunized with either of the following immunogens: H1 WT, H1+9+PEG. Before the primary immunization, serum samples were taken from each guinea pig to act as a preimmune control (SI Appendix Fig. 6C). For immunizations, 100 $\mu\text{L}$  of H1 WT (.5mg/mL), H1+9+PEG (.5mg/mL) in 1xPBS with 5% glycerol mixed 1:1. Guinea pigs were immunized intramuscularly with one of the immunogens and Imject Alum adjuvant (ThermoFisher) mixed 1:1 by volume. All immunizations were conducted at Josman LLC (Napa, CA). Serum was then isolated from the guinea pigs on day 14 post-immunization. The animals were boosted on day 20. The boost contained the same amount of immunogen as the primary immunization. Serum was harvested again on day 30 for all animals.

**Hemagglutinin ELISAs:** Plates were made by coating with 50  $\mu\text{L}$  of 5  $\mu\text{g/mL}$  HA antigens (H1 IZ, H2 IZ, or H5 IZ) in 50 mM sodium bicarbonate pH 8.75 for 1 hour. These were washed 3x with 300  $\mu\text{L}$  of ddH<sub>2</sub>O and blocked with 100 $\mu\text{L}$  Chonblock (Chondrex) for at least 1 hour at room temperature. For plates made without antigen, plates were initially activated with 50  $\mu\text{L}$  of 50mM sodium bicarbonate pH 8.75 for 1 hour and then washed 3x with 300  $\mu\text{L}$  of ddH<sub>2</sub>O and blocked with 100 $\mu\text{L}$  Chonblock (Chondrex) for at least 1 hour at room temperature. Following blocking, serum samples were added at 10x serial dilutions, incubated for 1 hour at room temperature, and then washed 3x with 1xPBST. An anti-guinea pig HRP secondary antibody (Abcam) was added at a 1:20,000 dilution in PBST, and the plates incubated for 1 hour at room temperature. The plates were then washed 6x with 1xPBST, developed for 12 mins using 1-Step Turbo TMB ELISA substrate solution (ThermoFisher), and quenched using 2M H<sub>2</sub>SO<sub>4</sub>. The readout of this colorimetric assay was determined using a 96 well plate reader (Biotek). Samples were baseline subtracted using the average of wells that were coated with antigen but only exposed to secondary antibody. Confidence intervals determined using Prism 7 (GraphPad). The EC<sub>25</sub> was calculated for each individual animal by doing a Sigmoidal, 4PL fit on Prism Graph Pad and solving for the unknown value of OD<sub>450</sub> = 0.60. OD<sub>450</sub> = 0.60 was selected as 25% of the maximum value of antibodies binding to H1 HA and assumed to be the same for all HA types based on the similarity of MEDI8852 positive control binding (SI Appendix Fig. 5B). The MEDI8852 positive control gives confidence that the same amount of antigen is plated on each ELISA plate. The resulting curve fit determined the EC<sub>25</sub>. Statistical significance determined

using an unpaired t test assuming Gaussian distribution and that both populations have the same SD. Analysis done on Prism 7 (GraphPad).

**Biolayer Interferometry on Pooled Animal Serum:** Pooled serum for each group was produced by combining equal volumes of serum from each animal. HIS1K tips were loaded at 50 nM per antigen to a 0.8 nm shift (2 per antigen, either H1 IZ, H2 IZ, or H5 IZ). Tips were then washed 2x and immersed in a 1:20 dilution of pooled serum (one antigen-loaded tip per pooled serum sample). Negative control tips (2) were also included. Therefore, tips containing H1 IZ, H2 IZ, H5 IZ, or naked tips, were all simultaneously incubated in pooled serum from either H1 WT immunized animals or H1+9+PEG immunized animals. The association with serum was left to run for 1 hour and then dissociation was left to run for 1 hour. The resulting curves were then baseline subtracted using the naked tip that was simultaneously immersed in serum.

Sequences:

H1 HA +9 LYS Protein (Purified)

DTICIGYHANNSTDTVDTVLEKNVTVTTHSVNLLLED SHNGKLCKLKGIAPLQLGKCSVAG  
WILGNPECELLISKESWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKES  
SWPNHTVKGVASCSHGKSSFYKNLLWLTGKNGLYPKLSKSYKNNKEKEVLVLWGV  
HHPPNIKNQKALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGD  
TIIFEANGNLIAPWYAFALSRGFGSGIITSNAPMDECDACKCQTPQGAINSSLPFQNVHPVTI  
GECPKYVRS AKLRMVTGLRNIPQRETGGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQ  
GSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGF  
LDIWTYNAELLVLENER TLDFHDSNVKNLYEKVKSQ LKNNAKEIGNGC FEFYHKCNN  
ECMESVKN GTYDYPKYSEESKLNREKIDGSGYIPEAPRDGQAYVRKDGEWVLLSTFLGS  
GLNDIFEAQKIEWHEGHHHHHH

H1 HA +9 LYS Protein (Expressed)

**MYRMQLLS**CIALSLALVTNSDTICIGYHANNSTDTVDTVLEKNVTVTTHSVNLLLED SHN  
GKLCKLKGIAPLQLGKCSVAGWILGNPECELLISKESWSYIVETPNPENGTCFPGYFADY  
EELREQLSSVSSFERFEIFPKESSWPNHTVKGVASCSHGKSSFYKNLLWLTGKNGLYP  
KLSKSYKNNKEKEVLVLWGVHHPPNIKNQKALYHTENAYVSVVSSHYSRRFTPEIAKR  
KVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFALSRGFGSGIITSNAPMDECDACK  
CQTPQGAINSSLPFQNVHPVTIGECPKYVRS AKLRMVTGLRNIPQRETGGLFGAIAGFIEG  
GWTGMVDGWYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGK  
EFNKLERRMENLNKKVDDGF LDIWTYNAELLVLENER TLDFHDSNVKNLYEKVKSQ L  
KNNAKEIGNGC FEFYHKCNN **ECMESVKN** GTYDYPKYSEESKLNREKIDGSGYIPEAPRD  
GQAYVRKDGEWVLLSTFLGSGLNDIFEAQKIEWHEGHHHHHH

**IL2 Signal Sequence**

*Avi Tag*

Foldon Tag

Hexa His-Tag

H1 HA WT (purified)

DTICIGYHANNSTDTVDTVLEKNVTVTHTSVNLLLED SHNGKLCLLKGIAPLQLGNCSVAG  
WILGNPECELLISKESWSYIVETPNPENGTCFPGYFADYEELREQLSSVSSFERFEIFPKES  
SWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYPNLSKSYVNNKEKEVLVLWGV  
HHPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRPKVRDQEGRINYYWTLLEPGD  
TIIFEANGNLIAPWYAFALSRGFGSGIITSNAPMDECDACKCQTPQGAINSSLPFQNVHPVTI  
GECPKYVRS AKLRMVTGLRNIPQRETGGLFGAIAGFIEGGWTGMVDGWYGYHHQNEQ  
GSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGKEFNKLERRMENLNKKVDDGF  
LDIWTYNAELLVLENERTLDFHDSNVKNLYEKVKSQ LKNNAKEIGNGC FEFYHKCNN  
ECMESVKNGTYDYPKYSEESKLNREKIDGSGYIPEAPRDGQAYVRKDGEWVLLSTFLGS  
GLNDIFEAQKIEWHEGHHHHHH

H1 HA WT (Expressed)

**MYRMQLLS**CIALSLALVTNSDTICIGYHANNSTDTVDTVLEKNVTVTHTSVNLLLED SHN  
GKLCLLKGIAPLQLGNCSVAGWILGNPECELLISKESWSYIVETPNPENGTCFPGYFADY  
EELREQLSSVSSFERFEIFPKESSWPNHTVTGVSASCSHNGKSSFYRNLLWLTGKNGLYP  
NLSKSYVNNKEKEVLVLWGVHHPNIGNQRALYHTENAYVSVVSSHYSRRFTPEIAKRP  
KVRDQEGRINYYWTLLEPGDTIIFEANGNLIAPWYAFALSRGFGSGIITSNAPMDECDACK  
CQTPQGAINSSLPFQNVHPVTIGECPKYVRS AKLRMVTGLRNIPQRETGGLFGAIAGFIEG  
GWTGMVDGWYGYHHQNEQGSGYAADQKSTQNAINGITNKVNSVIEKMNTQFTAVGK  
EFNKLERRMENLNKKVDDGFLDIWTYNAELLVLENERTLDFHDSNVKNLYEKVKSQ L  
KNNAKEIGNGC FEFYHKCNNECMESVKNGTYDYPKYSEESKLNREKIDGSGYIPEAPRD  
GQAYVRKDGEWVLLSTFLGSGLNDIFEAQKIEWHEGHHHHHH

**IL2 Signal Sequence**

*Avi Tag*

Foldon Tag

Hexa His-Tag

Medi8852 LC WT Protein (Purified)

DIQMTQSPSSLSASVGDRVTITCRTSQSLSSYTHWYQQKPGKAPKLLIYAASSRGSGVPSRFSGSGSGTDFTLT  
ISSLPEDFATYYCQQSRTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVCLNNFYPREAKVQWKVD  
NALQSGNSQESVTEQDSKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Medi8852 LC WT Protein (Expressed)

**MGWSCILFLVATATGVHSD**IQMTQSPSSLSASVGDRVTITCRTSQSLSSYTHWYQQKPGKAPKLLIYAASSR  
GSGVPSRFSGSGSGTDFTLTISLPEDFATYYCQQSRTFGQGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASV  
CLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYLSSTLTLSKADYEKHKVYACEVTHQGLSSPV  
TKSFNRGEC

**Signal Sequence**

Constant Region

Medi8852 HC Reduced Affinity Protein (Purified)

QVQLQQSGPGLVKPSQTLSTCAISGDSVSSYNAVWNWIRQSPSRGLEWLGATAYRSGWYNDYAESVKSR  
TINPDTSKNQFSLQLNSVTPEDTAVYYCARSGHITVFGVNVDAFDMWGQGTMTVSSASTKGPSVFPLAPS

SKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHK  
PSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVKFNWY  
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYT  
LPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKSRWQQGNV  
FSCSVMHEALHNHYTQKSLSLSPGK

Medi8852 HC Reduced Affinity Protein (Expressed)

**MGWSCIILFLVATATGVHS**QVQLQQSGPGLVKPSQTLSTCAISGDSVSSYNAVWNWIRQSPSRGLEWLGA  
TAYRSGWYNDYAESVKSRLINPDTSKNQFSLQLNSVTPEDTAVYYCARSGHITVFGVNVDADFDMWGQGT  
MVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSS  
VTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPE  
VTCVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKAL  
PAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSD  
GSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK

**Signal Sequence**

**Ala Mutation**

**Constant Region**



# Figure S1

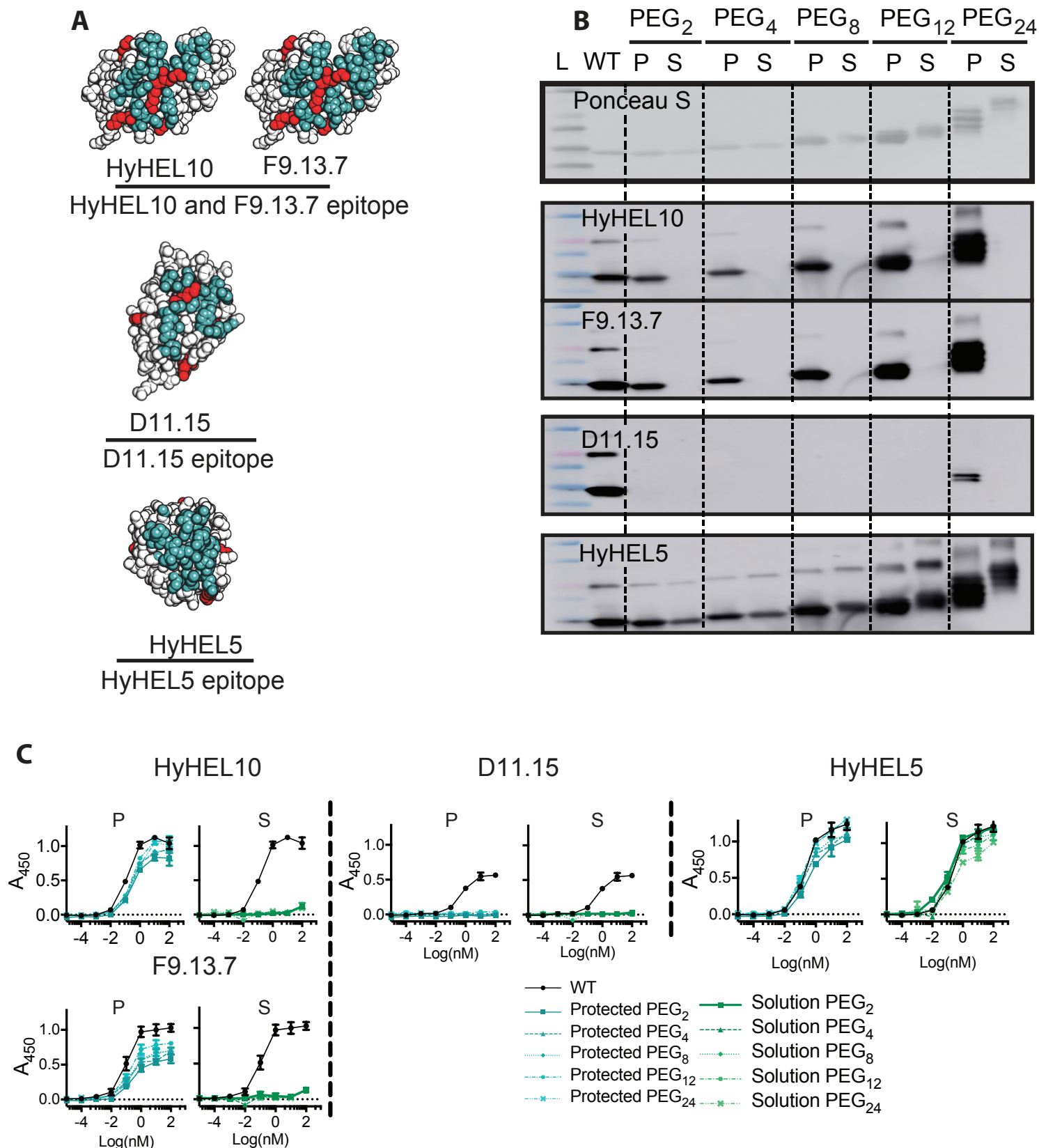


Figure S1: PMD with HEWL. (A) Epitopes of HEWL mAbs depicted in a face-on view. The epitope is colored blue and lysine residues red. The mAbs are HyHEL10 (3HFM), F9.13.7 (1FBI), D11.15 (1JHL) and HyHEL5 (1YQV). (B) PMD-generated HEWL antigens compared to PEGylated HEWL antigens generated without PMD. The mAb HyHEL10 was used as the PMD antibody and different PEG lengths (2, 4, 8, 12, and 24) were tested. The lanes marked P contain PMD-generated HEWL using HyHEL10, the adjacent lanes marked S contain HEWL PEGylated in solution (i.e., without mAb protection). L, molecular weight ladder and WT, HEWL. Top, protein stain (Ponceau S). Lower, western blots using the indicated mAb. The expected MW of WT HEWL is 14.3 kDa. Commercial sources are known to have a small fraction of dimer at 28.6 kDa (see methods). (C) ELISA analyses of PMD-generated HEWL antigens compared to PEGylated HEWL derivatives generated without PMD. The mAb used in the ELISA is indicated. P and S, same as above.

Figure S2

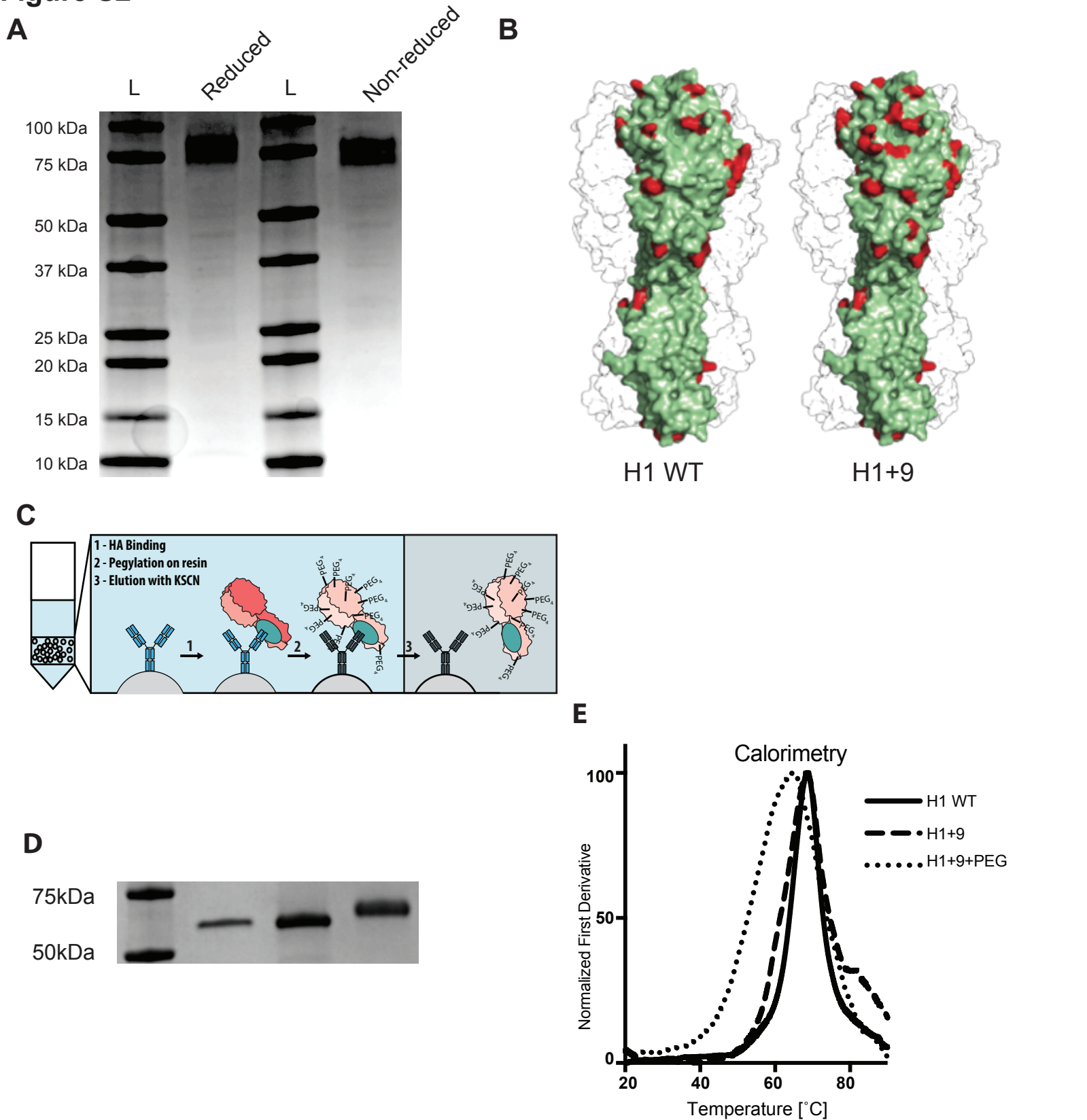


Figure S2: PMD with influenza HA. (A) SDS-PAGE gel analysis of reduced (lane 2) and non-reduced (lane 4) H1 WT protein. (B) Additional lysine residues added to H1 WT to produce H1+9 are shown on a space filling model of influenza HA. A single monomer is highlighted. Lysine residues are shown in red. Nine additional lysines were added at sites L60K, N71K, T146K, N155K, R162K, N176K, V182K, G202K, and R205K of the H1 HA protein. (C) PMD with influenza HA. In step 1, HA is added to beads (grey) to which the bnAb (dark blue) has been previously attached. The blue oval on the HA trimer denotes the target epitope on the stem and the red shading denote off-target regions of HA. In step 2, the chemical reaction to add PEG4 chains to the surface of HA is performed and the beads are washed. In step 3, the PMD HA trimer is dissociated. (D) SDS-PAGE gel comparing H1 WT (lane 2), H1+9 (lane 3), and H1+9+PEG (lane 4). All proteins were deglycosylated using PNGase treatment prior to analysis. Standard ladder shown in lane 1. (E) Thermal melt calorimetry comparing H1 WT, H1+9, and H1+9+PEG. Normalized first derivatives of the melt curves (Nanotemper) are shown.

**Figure S3**

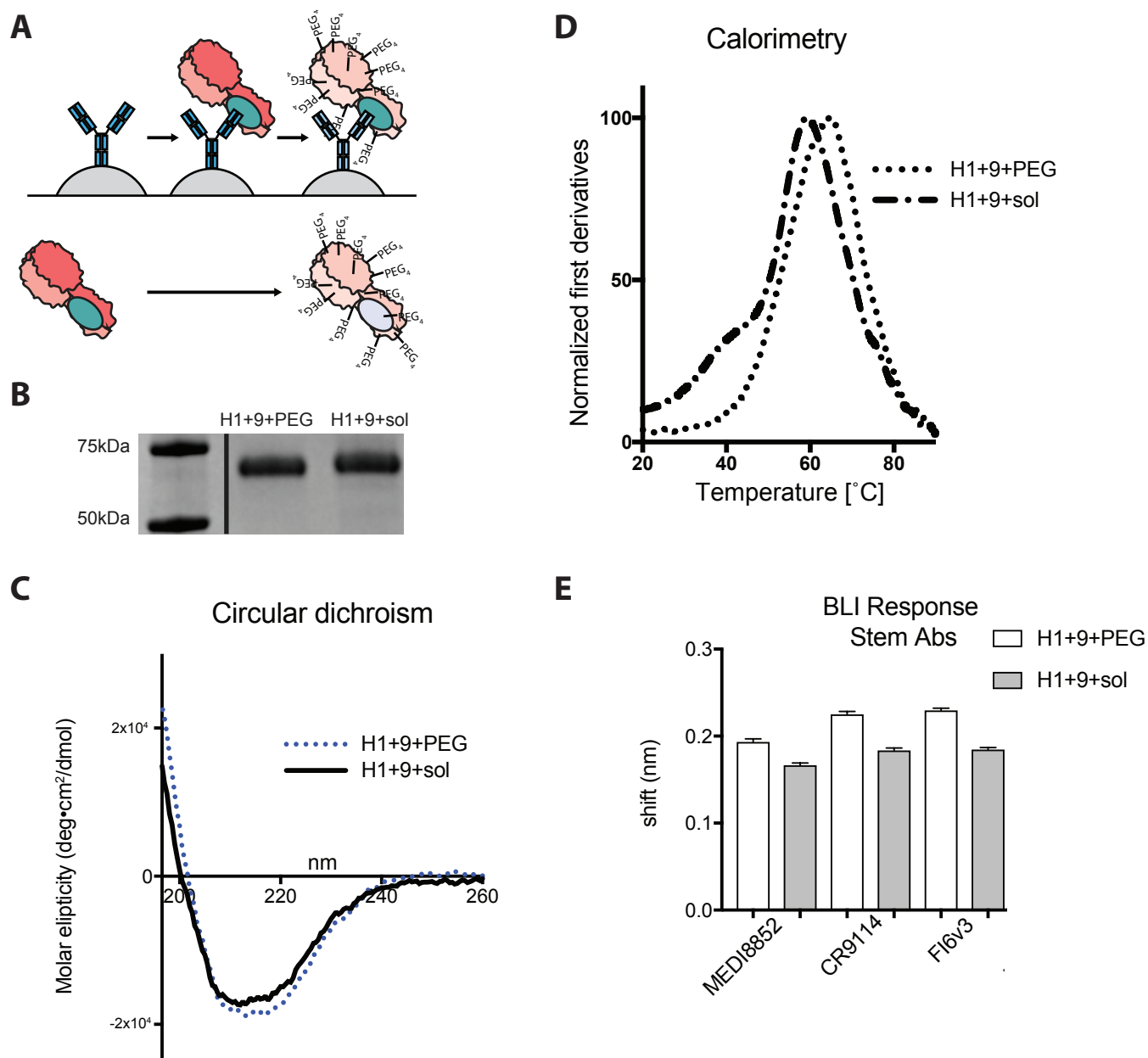


Figure S3: Comparison of HA PEGylated in solution (H1+9+sol) to HA PEGylated using the PMD protocol (H1+9+PEG). (A) A general schematic of the comparison between H1+9+PEG and H1+9+sol is shown. (B) SDS-PAGE comparing H1+9+PEG and H1+9+sol. Both proteins were deglycosylated using PNGase treatment prior to analysis. (C) Circular dichroism spectroscopy comparing the same 2 proteins. (D) Thermal melt calorimetry comparing the same 2 proteins. Normalized first derivatives of the melt curves are shown. (E) Binding of stem-directed mAbs measured by BLI. HIS1K tips were loaded to .22nm. The shift after 2 minutes of association is shown. Error bars represent the standard deviation of triplicate measurements.

Figure S4

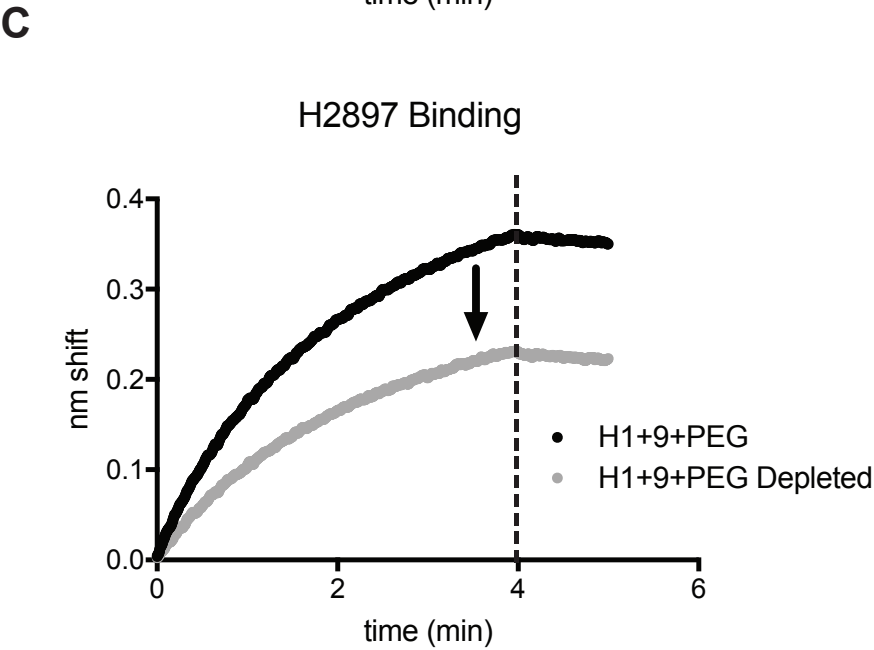
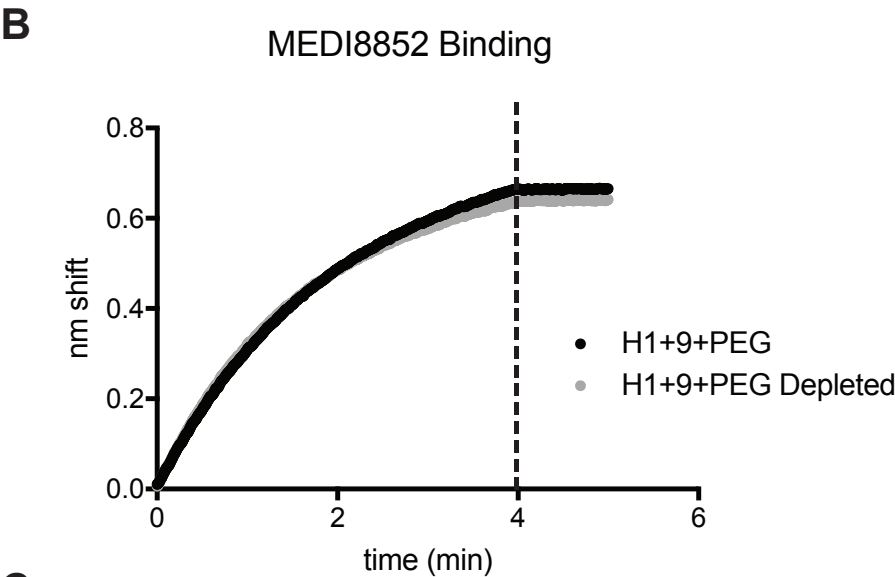
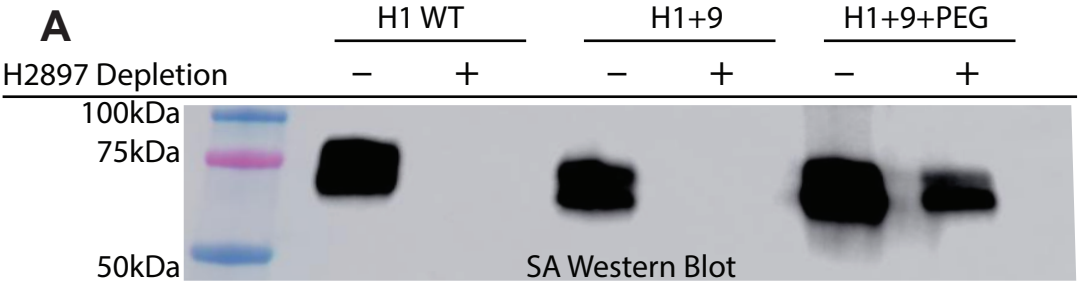


Figure S4: H1+9+PEG depletion with the H2897 mAb. (A) Samples of H1 WT, H1+9, and H1+9+PEG were either depleted (incubated with excess H2897) or not (incubated with PBS). Resulting samples were run on a streptavidin western blot to probe the extent of depletion. A standard MW ladder is shown in lane 1. (B) BLI binding of MEDI8852 to either H1+9+PEG un-depleted (lane 6, part A) or depleted with H2897 (lane 7, part A). To ensure an identical amount of protein was analyzed, a loading threshold was applied to the SA biosensors to .7nm. (C) BLI binding of H2897 to either H1+9+PEG un-depleted (lane 6, part A) or depleted with H2897 (lane 7, part A). A threshold of .7nm was utilized as above.



**Figure S5**

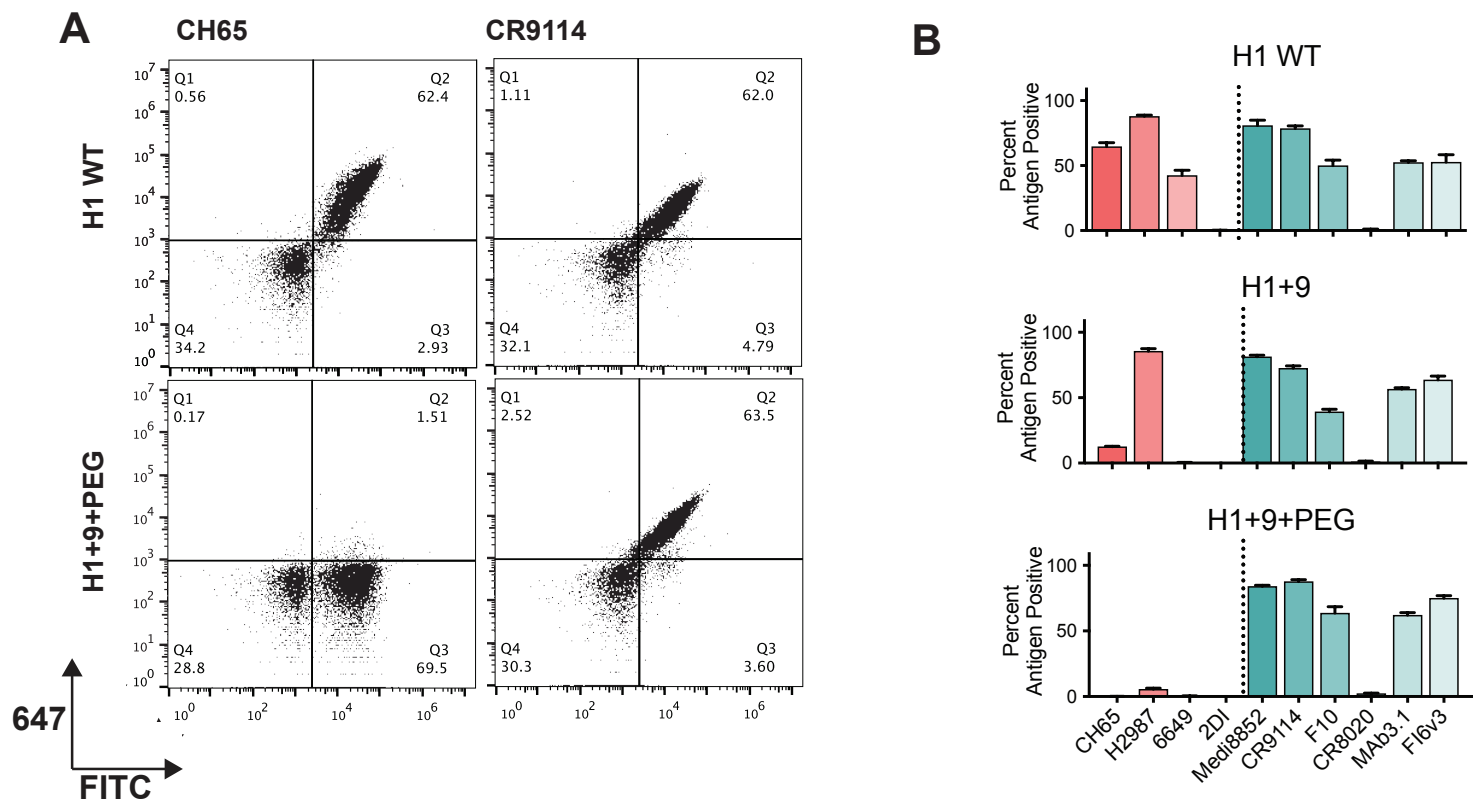


Figure S5: Yeast-displayed scFv clonal binding. (A) Four sample FACS plots of yeast displaying either a stem-directed scFv (CR9114, right plots) or a head-directed scFv (CH65, left plots). Yeast were sorted with either H1 WT (top plots), or H1+9+PEG (bottom plots) as tetramers. The top two quadrants on each plot are positive for antigen binding (647 positive) and the right two quadrants on each plot are positive for scFv expression (FITC binding). (B) Binding of yeast displaying scFvs of head mAbs (red) and stem mAbs (blue) sorted with either H1 WT (top), H1+9 (middle), or H1+9+PEG (bottom). Percent antigen positive is determined by dividing the count in the top right quadrant of FACS plots by the combined count of the right two quadrants, multiplied by 100.

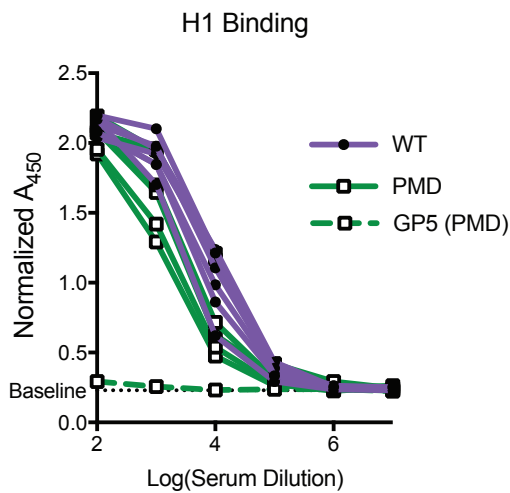
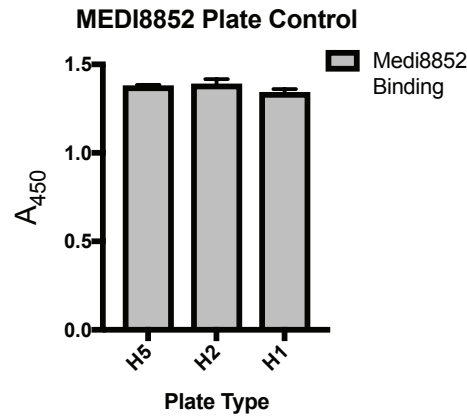
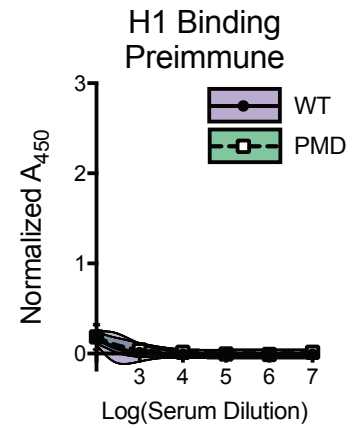
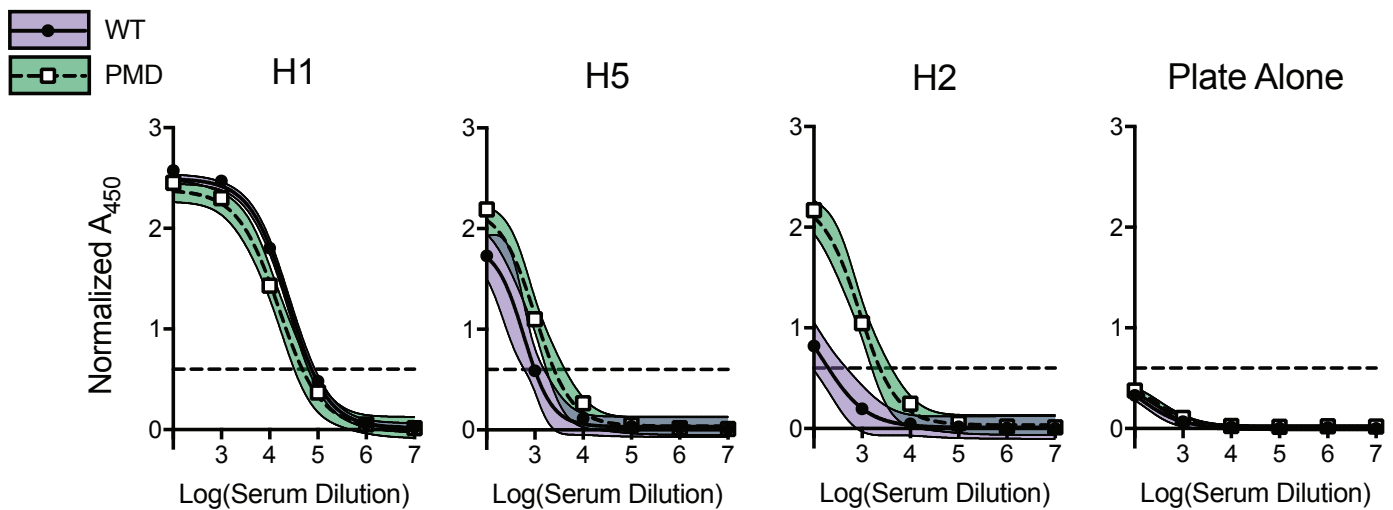
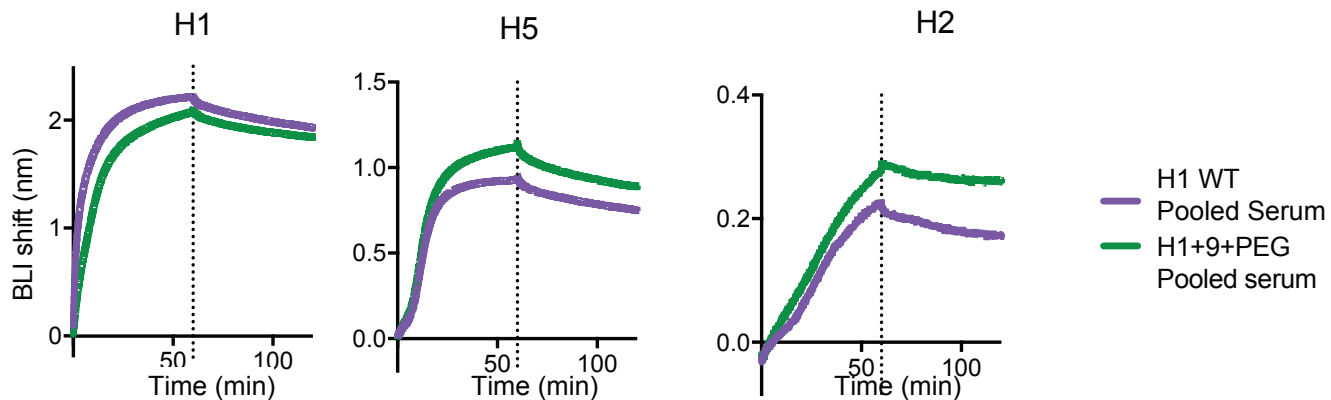
**Figure S6****A****B****C****D****E**

Figure S6: Analysis of antisera from guinea pigs immunized with H1 WT or H1+9+PEG. (A) ELISA results of all individual animals from Fig. 5 (main text) as well as Gp5 (in the PMD group), which was excluded from further analysis due to its weak binding. Baseline is the average absorbance of wells coated with antigen and treated with only secondary antibody (B) 100nM MEDI8852 (positive control) binding to ELISA plates used in Fig. 5 (main text) and SI Appendix Fig. 6D. This positive control is shown to demonstrate that a similar amount of antigen is coated on each plate regardless of the strain of HA. (C) Preimmune serum binding to H1 WT as measured by ELISA. (D) Binding of sera to HA ectodomain trimers from either H1, H2, H5 strains or blocking agent alone measured by ELISA. The values are presented as mean and 95% confidence interval. Dashed line shows the EC25 used in Fig. 5 (main text). ELISAs were conducted with a trimerization tag different than was used in immunization to avoid irrelevant antibody binding. The H2 HA protein used was derived from (A/Japan/305+/1957 (H2N2)) (Xu R, McBride R, Paulson JC, Basler CF, Wilson IA (2010) *J Virol* 84(4):1715–1721.) and the H5 HA protein used was derived from (A/Viet Nam/1194/2004 (H5N1)) (Govorkova EA, et al. (2005) *J Virol* 79(4):2191 LP-2198.) with a linker-IZ-His tag construct. (E) BLI analyses of pooled serum from animals immunized with H1 WT or H1+9+PEG. Biosensors were coated with HA ectodomain trimers from either H1, H2 or H5 strains at the same threshold. Binding (association) and dissociation were each measured for approximately one hour in order to evaluate a wide range of antibody affinities in the serum samples. This experiment was replicated with similar results.

## References:

- 1) Thomas BR, Vekilov PG, Rosenberger F (1996) Heterogeneity Determination and Purification of Commercial Hen Egg-White Lysozyme. *Acta Crystallogr Sect D* 52(4):776–784
- 2) Whittle JR, et al. (2014) Flow cytometry reveals that H5N1 vaccination elicits cross-reactive stem-directed antibodies from multiple Ig heavy-chain lineages. *J Virol* 88(8):4047–4057.
- 3) Han P, et al. (2014) A long noncoding RNA protects the heart from pathological hypertrophy. *Nature* 514(7520):102–106.