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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at regions outside the epitope of interest. Finally, cross-strain boosting uses sequential immunizations with other strains or chimeric proteins that vary at off-target epitopes.

Significant progress has been made with these immunofocusing strategies. These methods have inherent limitations, however. They are not easily generalizable, making application to new antigens challenging. With the exception of epitope scaffolding (which requires extensive protein engineering), these immunofocusing methods are also generally "low-resolution" (i.e., directed toward a region of the protein that is significantly larger than a typical Ab epitope). In addition, with some of these methods, maintaining the precise 3D structure of the epitope can be challenging.

Here we introduce a method, which we call "protect, modify, deprotect" (PMD), that has the potential to provide high-resolution immunofocusing in a generalizable manner with minimal protein engineering. The method uses a bnAb as a molecular stencil to generate an antigen that focuses the immune response toward the bnAb epitope. Although bnAbs have been used previously to inform and guide immunogen design, we are not aware of their use as reagents in the creation of vaccine candidates.

The steps in PMD are (*i*) protection of an epitope on an antigen by binding of a bnAb, (*ii*) chemical modification of exposed sites to render them nonimmunogenic, and (*iii*) deprotection of the epitope of interest by dissociation of the Ab-antigen complex. This produces an immunogen in which the only unmodified region is the epitope mapped by the bnAb (Fig. 1).

To establish the PMD method, we use hen egg white lysozyme (HEWL), a stable monomeric protein with well-characterized epitopes (44). We protect an epitope on HEWL by binding it to an mAb-conjugated resin (45). We then modify surface amines to add short polyethylene glycol (PEG) chains, which are known to decrease immunogenicity locally (28, 46–48). The modified HEWL derivatives, dissociated from the mAb resin, have antigenic properties consistent with the expected properties based on the location of surface amines in Ab cocrystal structures.

We then use PMD to generate an influenza HA antigen designed to skew the immune response toward a conserved epitope on the stem. We confirm that the PMD-generated HA is properly folded and displays markedly reduced binding to mAbs that target the HA head, while maintaining binding to mAbs that target the stem. We also use the PMD-generated HA as bait in fluorescence-activated cell sorting (FACS) experiments with a polyclonal yeast mini-library displaying single-chain variable fragments (scFvs) and obtain significant enrichment for stem-directed clones. Finally, antisera from guinea pigs immunized with this PMD-generated HA show a skewed immune response toward the stem, as demonstrated by a more cross-reactive Ab response compared with antisera obtained from animals immunized with unmodified HA.

Results

Establishing the PMD Method with HEWL. The initial validation of the PMD method was done using HEWL, a well-characterized protein with known antigenic epitopes (Fig. 2*A*). We chose to use amine-reactive *N*-hydroxysuccinimide (NHS) esters as our modifying reagent, because NHS-esters rapidly react with lysine residues and the N-terminal amino group at neutral pH (Fig. 2*B*). There are three major nonoverlapping, conformation-dependent epitopes on HEWL mapped by mAbs: HyHEL10 (49) and F9.13.7 (50), D11.15 (51), and HyHEL5 (52) (Fig. 2*A* and *SI Appendix*, Fig. S1*A*). Cocrystal structures are available for each of these complexes. The epitope mapped by HyHEL10 and F9.13.7 contains two lysine residues, K96 and K97 (*SI Appendix*, Fig. S1*A*). D11.15 binds over a different lysine residue, K116. Finally, HyHEL5 does not contain any reactive amines (lysine residues or the N terminus) in its epitope (*SI Appendix*, Fig. S1*A*).

We selected HyHEL10 as the protecting mAb for our proof-of-concept PMD study because (*i*) it is bound over two lysine residues; (*ii*) it shares a significant portion of its epitope with F9.13.7, allowing for a separate test of epitope protection; and (*iii*) it does not contain the lysine residue present in the D11.15 epitope.

During the deprotection step in PMD, there is a need to separate the modified antigen from the protecting mAb. To facilitate this separation, we conjugated HyHEL10 to resin. We determined that HEWL bound to this HyHEL10 resin can be eluted at low pH (100 mM glycine, pH 1.5). Low pH elution might be not be suitable in other cases; indeed, a neutral pH elution was used with HA, as discussed below.

For the modification step, we investigated PEG chains of differing lengths using NHS-polyethylene glycol$_n$-methyl (NHS-PEG$_n$-me), where n denotes the number of ethylene glycol units (n = 2, 4, 8, 12, or 24) (Fig. 2*B*). HEWL antigens that were PEGylated on an HyHEL10 resin and then dissociated (following the PMD protocol) are referred to as HEWL-pro$_n$. We simultaneously produced HEWL antigens that were PEGylated in solution without Ab protection, and refer to these as HEWL-sol$_n$.

PMD-HEWL Decreases Antigenicity at Off-Target Sites While Maintaining On-Target Antigenicity. Using biolayer interferometry (BLI), we compared the binding of the four mAbs described above to wild-type (WT) HEWL, the five HEWL derivatives PEGylated on HyHEL10 resin, and the five HEWL derivatives PEGylated in solution. BLI measures the kinetics of protein–protein interactions and allowed us to determine dissociation constants (K_D) for these 44 interactions.

The $-\log(K_D)$ values with an overlaid heat map are shown in Fig. 2*C*. The top two rows of the heat map show that both HyHEL10 and F9.13.7 do not bind to HEWL-sol$_n$ antigens, presumably because modifying the lysine residues K96 and K97 in their epitopes interferes with binding. Interestingly, HEWL-sol$_2$ does not ablate HyHEL10 binding, suggesting that PEG$_2$ is too short to fully disrupt Ab binding, while PEG$_4$ is sufficient. Conversely, the same two Abs, HyHEL10 and F9.13.7, retain their binding to HEWL-pro$_n$ antigens (Fig. 2*C*). This demonstrates that immobilization of HEWL on a HyHEL10 resin during PEGylation sufficiently protects this conformation-dependent epitope from modification.

HyHEL5 binds to all HEWL derivatives (Fig. 2*C*). There are no amines within the epitope for this mAb. These results indicate that PEGylation of amines on the protein with chains up to PEG$_{24}$ does not interfere with binding of HyHEL5. It is possible that longer PEG chains would have an effect (cf. ref. 53). We refer to such epitopes that retain their antigenicity even after the PMD protocol as "antigenic holes."

Finally, D11.15 binds to the WT protein but does not bind to any of the PEGylated proteins. D11.15 binds over a lysine residue outside of the HyHEL10 epitope. Thus, PMD can effectively modify antigenic sites outside the epitope of interest. ELISAs

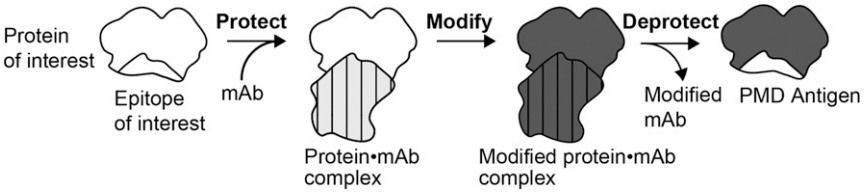

Fig. 1. A general schematic of the PMD strategy. First, the epitope is protected by combining the mAb (hashed) with the antigen (white). Then the surfaces of the protein complex are modified to render them nonimmunogenic (shown as darker shading). Finally, the epitope is deprotected by removal of the mAb.

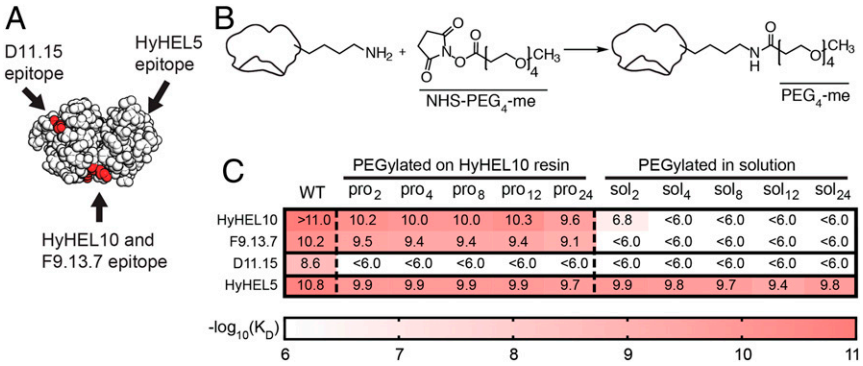

Fig. 2. PMD with HEWL. (*A*) HEWL structure with the epitopes of D11.15, HyHEL10, F9.13.7, and HyHEL5 indicated by arrows and lysine residues in red. (*B*) NHS-ester reaction with a lysine residue is shown with NHS-PEG_4-me. (*C*) Binding of mAbs to HEWL antigens PEGylated in solution (without PMD) or to PMD-generated HEWL antigens created with mAb HyHEL10 as the protecting Ab and using different PEG_n 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. S1*C*).

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. S1*B*). The results are generally consistent with those obtained by BLI (Fig. 2*C*); however, these analyses reveal a "laddering" phenomenon that is particularly prominent when longer PEGylation reagents are used (*SI Appendix*, Fig. S1*B*). 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_n 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 ε-amino or α-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 HAΔSA, 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. S2*A*) 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. S2*B*).

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. S2*C*). H1+9 was bound to MEDI8852* resin and the complex was PEGylated with NHS-PEG_4-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. S2*D*). Indistinguishable CD spectra for H1 WT, H1+9, and H1+9+PEG suggest that these proteins have the same folded structure (Fig. 3*A*). The gel filtration results for all three proteins are consistent with those expected for a trimer (Fig. 3*B*), 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. S2*E*). 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. S3*A*). H1+9+sol has a slightly higher molecular weight than H1+9+PEG as determined by SDS/PAGE analysis (*SI Appendix*, Fig. S3*B*), 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. S3*C*). H1+9+sol melts at a lower temperature than H1+9+PEG, with an apparent pretransition, as determined by calorimetry (*SI Appendix*, Fig. S3*D*). 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. 3*C*). All six Abs bound to H1 WT (Fig. 3*C*, *Left*). Stem-directed Abs retained their binding after lysine substitution (H1+9) and after PEGylation (H1+9+PEG) (Fig. 3*C*, *Right*, blue). This demonstrates

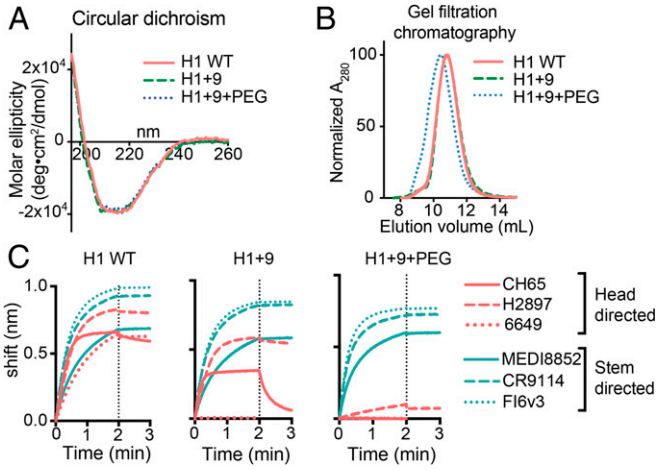

Fig. 3. PMD with influenza HA. (*A*) CD spectroscopy comparing H1 WT, H1+9, and H1+9+PEG in 0.25× PBS. (*B*) Gel filtration analysis comparing H1 WT, H1+9, and H1+9+PEG FPLC in 1× PBS with a Superdex 200 Increase column. (*C*) Binding of anti-HA mAbs to H1 WT, H1+9, and H1+9+PEG as measured by BLI (head 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. 3*C*, 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. S4*A*). Furthermore, the H1+9+PEG protein that was not depleted by our procedure showed decreased binding to H2897 with retention of binding to MEDI8852 (*SI Appendix*, Fig. S4 *B* and *C*). Thus, the H2897 hole apparent in Fig. 3*C* 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. S3*E*). 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 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. S5*A*. 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. S5*B*). 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 Imject 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. S6*A*) 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. S6*D*, *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. S6*D*). The difference is even more pronounced with binding to H2 HA (*SI Appendix*, Fig. S6*D*). 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. S6*E*). Taken together,

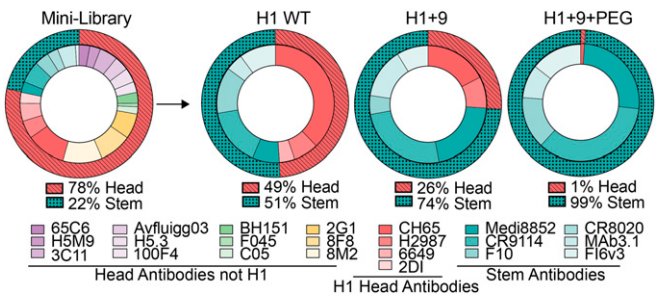

Fig. 4. FACS of a mini-library of yeast-displayed scFvs using H1 WT, H1+9, or H1+9+PEG as the selection bait. The outer ring depicts the relative amounts of head- and stem-binding scFvs, and the inner ring represents the abundance of sequenced clones for the mini-library (*Left*) and antigen-selected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

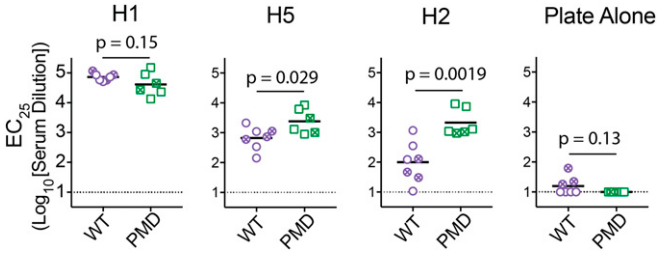

Fig. 5. Cross-reactive binding of antisera from guinea pigs immunized with H1 WT (purple circles; $n = 7$) or H1+9+PEG (green squares; $n = 6$). ELISA results (log EC25) for binding to H1, H2, or H5 HA trimers are depicted from two separate experiments, indicated as symbols with crosses and open symbols. 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 PBSM 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 (Miltenyi Biotec) for 15 min, washed twice, and then flowed.

Guinea Pig Immunizations. Here 50 μg of each immunogen (either H1 WT or H1+9+PEG) in 100 μL 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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