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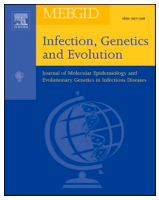

Research paper

Evolutionary dynamics of epitopes of limited variability on the head of influenza H1 haemagglutinin

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ABSTRACT

It is commonly assumed that naturally protective targets of immunity in influenza are highly variable. Theoretical work suggests, by contrast, that influenza evolution is primarily driven by naturally protective responses against epitopes of limited variability (ELV). At least one ELV has been identified on the head region of haemagglutinin of H1 influenza, opening up the possibility of producing a universal influenza vaccine. Here, for the first time, we provide a comprehensive catalogue of ELVs within the head region of H1 haemagglutinin and explain how they arise within its apparently high variable landscape. We show that the head region of H1 haemagglutinin can be decomposed into a number of discrete variable regions (VRs): ELVs tend to include a limited number of VRs compared to other epitopes either because of the smaller footprint of the associated antibody or because they are centred on VRs that are relatively isolated from others. Thus, the variability of an antibody binding site is determined by the number of variable residues included in its footprint rather than the intrinsic entropy of any particular region.

1. Introduction

Influenza is currently estimated to cause between a quarter to half a million deaths annually across the globe, with the majority occurring in those over 65 years of age (Paget et al., 2019). However, its precise impact has yet to be characterised in many parts of the world such as Africa, where much of the mortality and morbidity is concentrated in young children (Wang et al., 2020). The risk of complications from influenza infection also increases during pregnancy and among those with underlying medical conditions such as HIV, TB and certain immunodeficiencies (Krammer et al., 2018).

Efforts to reduce the burden of influenza through vaccination are complicated by the ability of the virus to mutate its naturally protective targets of immunity, particularly the B-cell epitopes situated on the head of the haemagglutinin (HA) surface glycoprotein which elicit neutralising antibodies (Krammer et al., 2018). Thus, while both inactivated and live-attenuated vaccines are made available each year, their protective efficacy can be compromised by a mismatch with the circulating strains. Vaccine effectiveness rarely exceeds 50%, falling in some years to as low as 10% (Wei et al., 2020), and correlations between vaccine coverage and influenza associated deaths appear to be weak (Paget et al., 2022). To address these issues, efforts are being made both to improve methods of production as well as to attempt to develop a "strain-transcending" universal influenza vaccine.

Current approaches towards developing a universal influenza vaccine typically rely on the possibility of artificially inducing protection against invariant targets of immunity (Wei et al., 2020) which are not naturally protective, such as B-cell epitopes situated on the haemagglutinin stalk (Puente-Massaguer et al., 2023; Bliss et al., 2022) or T-cell epitopes on conserved internal antigens (Del Campo et al., 2019; Evans et al., 2022). Recently, efforts have been made to artificially boost broadly neutralising antibodies against targets on the head of haemagglutinin (Leggat et al., 2022), which are very rarely produced during natural infection.

These approaches are generally predicated on the assumption that all naturally protective targets of immunity are highly variable. However, while it is necessarily true that all invariant targets of immunity must be poorly protective or very rarely generated upon natural infection (as, otherwise, influenza would be like measles where a single infection would confer lifelong immunity), the converse does not hold. In other

Abbreviations: HA, haemagglutinin; VRs, variable regions; ABS, antibody binding site; ELV, epitopes of limited variability; AA, amino acids; AAR, amino acid residues; CDR, complementarity-determining region; RBS, receptor-binding site.

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words, it is not necessarily true that all protective targets are highly variable. Indeed, we have demonstrated, through a combination of theoretical and experimental studies (Gupta et al., 1998; Recker et al., 2007; Thompson et al., 2018), that certain naturally protective targets of immunity located on the head of the haemagglutinin protein are of limited variability. This opens up the possibility of making vaccines providing broad coverage without sacrificing immunogenicity ie. multivalent vaccines of durable potency with a deployable number of allelic variants.

We have identified and characterised in vitro and in vivo at least one naturally protective epitope of limited variability (OREO) of the H1 subtype of influenza A (Thompson et al., 2018) in the head region of haemagglutinin (HA) proximal to the receptor binding site; we have performed proof-of-concept vaccine studies using the OREO epitope and are currently extending this work to identify epitopes of limited variability (ELV) in other subtypes of influenza A, as well as the Yamagata and Victoria lineages of influenza B with the aim of developing a universal vaccine against human influenza with the potential to extend to avian subtypes.

Here, we report further results of a protein sequence and structural bioinformatic exercise suggesting that the head region of H1 haemagglutinin can be viewed as a mosaic of variable regions (VR), and the hyper-variability observed among certain epitopes is a consequence of their footprint spanning several VRs, while existing epitopes of limited variability (ELV) tend to contain a very low number of VRs. This provides a novel explanation for how ELVs can exist alongside hypervariable epitopes on the haemagglutinin surface, and endorses the strategy of training the immune response to focus on ELVs to achieve universal protection.

2. Results

2.1. The OREO epitope in H1 haemagglutinin is a mosaic of two variable regions, VR1 and VR2

We have previously reported (Thompson et al., 2018) that H1 haemagglutinin contains an epitope of limited variability (ELV), OREO, spanning positions 146–159 (linear numbering) in its head region,

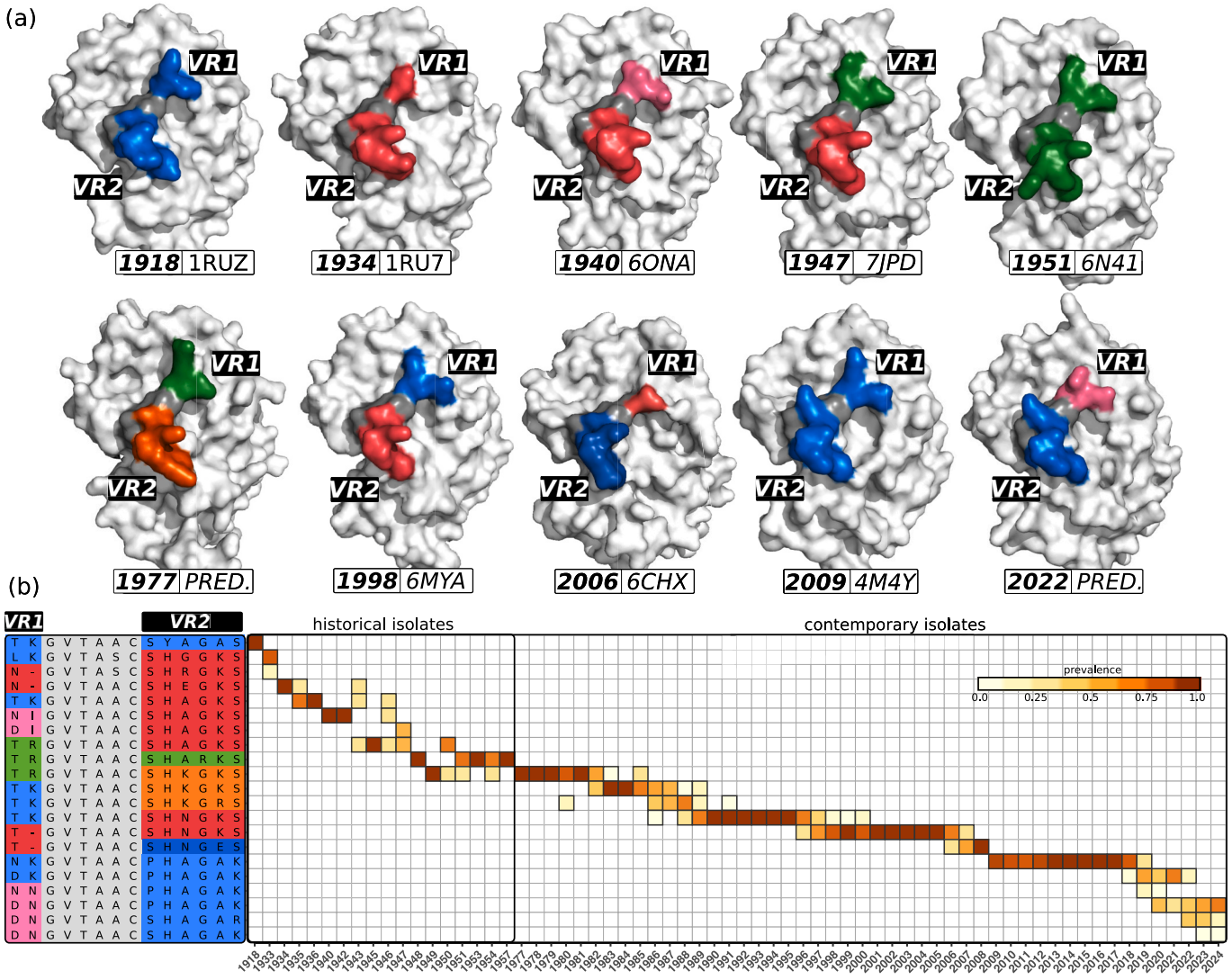

Fig. 1. The evolution of the H1 OREO epitope from 1918 to the present. (a) The two variable regions, VR1 and VR2, of OREO are tracked through time within a set of representative strains (year | crystal structure): the top row contains historical strains from the period 1918 to 1951 while the bottom row contains strains from the contemporary period (since 1977). Subtypes of VR1 and VR2 are differentiated by colours (VR1: blue, red, pink, green; VR2: blue, red, green and orange) and flank an invariant region shown in grey. (b) Prevalence of different combinations of VR1 and VR2 (constituting OREO) by year among historical and contemporary isolates (across all available sequences in GENBANK, up to December 2024). It should be noted that the number of isolates available varies significantly in time due to sampling bias. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which may be grouped into five types (originally termed red, blue, green, orange and pink) on the basis of the biochemical characteristics of residues in positions 147, 156, 158 and 159.

The antigenic relationships between these variants have also been interrogated through pseudotype neutralisation assays performed on mice vaccinated via a prime-boost-boost design with each variant presented on three different avian haemagglutinin scaffolds and subsequently challenged with PR8 (containing red OREO) and A/Cal/04/2009. Such past work (Thompson et al., 2018) revealed cross-reactivity between various pairs of variants, and notably also cross-protection against challenge between red and green OREO variants. This is not what would be expected if the two variants were recognized discretely, as suggested by the underlying theoretical framework, and thus poses a conundrum.

A novel explanation for these results is that OREO itself is a mosaic of two components which may be recognized separately or together, depending on the footprint of the antibody. That is, OREO can be deconstructed into two variable regions, VR1 and VR2, on either end of a conserved region both in sequence space and on the structure of HA (Fig. 1).

The residues 146 and 147 together comprise VR1 which can adopt 4 subtypes, although it could be argued that the blue and green subtypes are broadly equivalent with a positive charge R/K in position 147; the red subtype contains a deletion in 147 and the pink subtype is either negatively charged or neutral. VR2 contains residues 154–158, and also can be categorised into 4 subtypes, designated as red, blue, green and orange in Fig. 1. However, the orange and red variants show strong structural similarity, which is in agreement with the cross-reactivity and cross-protection observed in our previous mouse studies. The range of variation in OREO is determined by the possible combinations of VR1 and VR2. For example, the current circulating H1 variant (since-2020) is a chimera of a pink VR1 and a blue VR2.

2.2. VR2 constitutes a discrete region of diversity that is relevant for a variety of epitopes

We attempted to place VR1 and VR2 in the context of the diversity exhibited across the head region of HA. We collected variable residues

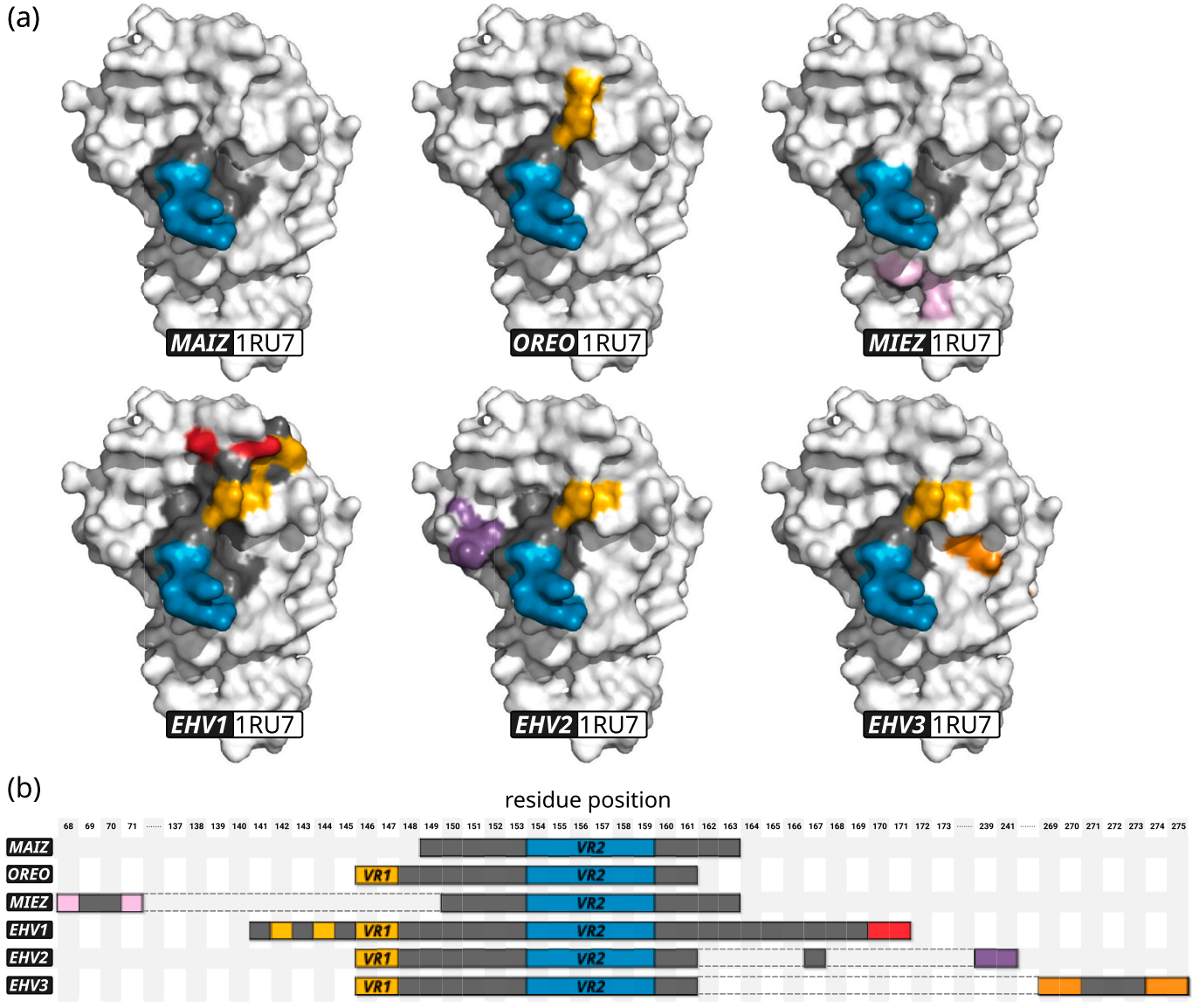

Fig. 2. Examples of epitopes containing the VR2 region. Antibody binding sites of equal footprint size (here, 800 Å and accessibility 10) positioned on VR2 can contain a variety of additional variable residues as shown here both (a) on the structure of haemagglutinin and (b) within sequence space. MAIZ, OREO and MIEZ constitute epitopes of limited variability (ELV). By contrast, EHV1–3 represent epitopes of high variability and include residues from three distinct variable regions. Colours correspond to variable residues as defined in Table S1.

that were close within the sequence into different colour groups as shown in Table S1. Within this scheme VR1 could be seen to belong to a larger subgroup containing variable residues 137,138,142,144,146 (shown in yellow in Table S1) while VR2 clearly constituted a stand-alone region of diversity spanning positions 156–159 (turquoise in Table S1) flanked by conserved regions comprising residues 148–155 and 160–169 (163 toggles between K and R).

Fig. 2 shows a range of epitopes containing VR2 as revealed by a structural bioinformatic mapping performed on PR8 (crystal structure IRU7, A / Puerto Rico / 8 / 1934), using an antibody binding site diameter of 800 Å. The OREO epitope, as defined in the previous section, contains both VR2 and VR1 connected by an invariant region marked in grey. Of even lower variability is an epitope we designate MAIZ, which contains only VR2, flanked by two invariant regions. Another ELV we can identify by these means is MIEZ containing VR2 and two variable residues in positions 68 and 71. By contrast, EHV1, EHV2 and EHV3 include a number of other variable residues in addition to both VR2 and VR1 which increases their potential variability. It should be noted that EHV2 maps closely to the Ca1 region within the original operational antigenic map of the HA molecule (Gerhard et al., 1981; Caton et al., 1982) obtained in the early '80s by comparative analysis of PR8 mutant viruses probed with monoclonal anti-HA antibodies.

2.3. *The variability of an antibody binding site (ABS) is determined by the number of variable regions (VR) included in its footprint*

A general principle emerging from our analysis is that binding-site variability is primarily driven by how many variable residues fall within the epitope footprint, rather than by the intrinsic entropy of any single region. Epitopes of limited variability (ELVs) will thus cluster around VRs which are spatially disjoint from other VRs, such as the VR2 region (shown in turquoise in Figs. 2–3) discussed in the previous section. By contrast, the variable region shown in red in Fig. 3 is in close proximity of blue and yellow VRs, as well as the purple VR region of an adjoining monomer; it is therefore difficult for an antibody to include elements of the red VR without also encompassing at least one of these other VRs. This is illustrated in Fig. 3d by highlighting the variable residues contained within a representative selection of antibody binding sites (ABS) of diameter 800 Å (each line represents an ABS with centroid in the position indicated in bold on the left).

ELVs, including those containing VR2 already shown in Fig. 2, are marked on the two-dimensional representation ABS map of Fig. 3d, with corresponding names. Each of these ELVs has the feature that they are principally focused on a single VR: INDY is primarily composed of the dark green VR (residues 185–187), RYEL is predominantly composed of the blue VR (residues 203, 204, 206, 210–213), while PIGG incorporates 2 contiguous VRs, light pink (residues 60, 64, 68) and dark pink (residues 289, 290, 293, 295). In line with the crystal structures shown in Fig. 3a-c, it is difficult to identify any ELVs associated with the red VR residues as they are typically found in conjunction with other VRs within the ABS. By contrast, ABS centred upon VR2 tend to remain of limited variability even when the scope of potential included residues is expanded by virtue of higher ABS diameter or reduced amino acid accessibility (Supplementary Figs. S2–3).

3. Discussion

In this paper, we introduce a novel framework in which the variability of an epitope arises as a combinatorial property of the variable regions contained within its footprint. Within this framework, the sequence space of the head region of HA is broken down into discrete variable segments (or regions, VR). Most of these regions show limited variability in the combinations of constituent amino acids as may be expected under the biochemical constraints within which they have to be assembled and perform their functions (Table S1). Many of these VRs are, however, in close proximity, if not contiguous, within the structure of the HA molecule In consequence, the footprint of a normal antibody is unlikely to be restricted to a single VR, thereby giving rise to a large combinatorial range of potential "epitopes". For example, if a particular VR can only exist in 5 possible conformations and another nearby VR can exist in 4 possible conformations, then an antibody binding site that spans both will have 20 different structural variations.

How, then, do ELVs arise? One possibility is that they are the targets of antibodies of smaller footprint than the typical 700–900 Å. Small footprint size may be achieved by the selective dominance of a single complementarity-determining region (CDR) loop as has been demonstrated for monoclonal antibodies such as CO5 (Ekiert et al., 2012; Ghafoori et al., 2023) and CH65 (Whittle et al., 2011). Both contain the VR1 portion of OREO as well as the final residue of VR2. Neutralisation patterns against H1 indicate that CO5 recognises strains with lysine in position 147 while CH65 is effective against strains with a deletion in position 147 and unable to neutralise strains with either a lysine or arginine in this position. These results are in agreement with the analysis of the OREO epitope presented in Fig. 1 and are supported by neutralisation patterns of other "broadly neutralising" monoclonal antibodies such as 5 J8 (Krause et al., 2011) and IFI which also include position 147 (Tsibane et al., 2012). Another recently identified monoclonal antibody (Li et al., 2022), C12H5, which includes the entire VR2 described in this study, exhibits binding and in-vitro microneutralisation patterns which are strongly consistent with our classification of VR2 into red and blue subtypes (Fig. 1). While C12H5 shows binding across a range of strains, this is much reduced for those with red VR2 and does not manifest in neutralising activity. By contrast, binding to strains with blue VR2 is high, leading to successful neutralisation.

These monoclonal antibodies exhibit extensive (even heterosubtypic) cross-neutralising properties on account of including only a limited number of residues surrounding the receptor binding site (RBS) (Table S1). As such, they constitute ELVs rather than broadly neutralising antibodies (bNabs) by virtue of their smaller footprints. By contrast, the antibody binding sites pivoting upon VR2 (Fig. 2) tend to be of limited variability, even when their footprint is large, owing to the relative isolation of VR2 which is flanked by conserved regions within the structure of HA, even though it sits upon the lip of the crater which contains the RBS. Antibody binding sites centred on VR2 may extend to contain some elements of other VRs and yet still retain low overall variability, as exemplified by the epitope OREO which we have identified as a potential vaccine candidate (Thompson et al., 2018).

Of the classical antibody binding sites previously described (e.g. (Thompson et al., 2018; Gerhard et al., 1981; Caton et al., 1982; Xu et al., 2010)), only Cb is composed of a single VR (light pink in Fig. 3 and Table S1) which can be resolved into 5 distinct variants. However, we could not find any ABS in our structural bioinformatic analysis (Fig. 3) which included only Cb on account of its close proximity to the other pink VRs. The sites Sa, Sb and Ca (as defined in (Xu et al., 2010)) include more than one VR and therefore exhibit higher variability, although with clear evidence of periodic re-emergence of the same variants. An extensive epitope mapping exercise using escape mutants by Matsuzaki et al. (Matsuzaki et al., 2014) confirms that the bulk of antibodies are restricted to the Sa, Sb and Ca2 (a part of Ca that includes VR2) but they were also able to identify at least one antibody which included Ca2 and position 147 (corresponding to OREO) and also a "broadly neutralising" monoclonal antibody (n2) centred on the RBS similar to those described above (Ekiert et al., 2012; Ghafoori et al., 2023; Krause et al., 2011; Tsibane et al., 2012). By contrast, the monoclonal antibody 2D1, derived from B-cells of survivors of the 1918 H1N1 pandemic (Xu et al., 2010), is highly diverse on account of possessing a footprint that spans 5 VRs; consequently it has low affinity for all strains except A/Cal/04/2009 which is identical in every single residue.

Our analysis challenges the idea that low variability is an indication of low natural potency (Fig. 4). Instead, we suggest that epitopes of low variability can be equally as protective as those of high variability, and therefore under equally high selection pressure. Our framework explains

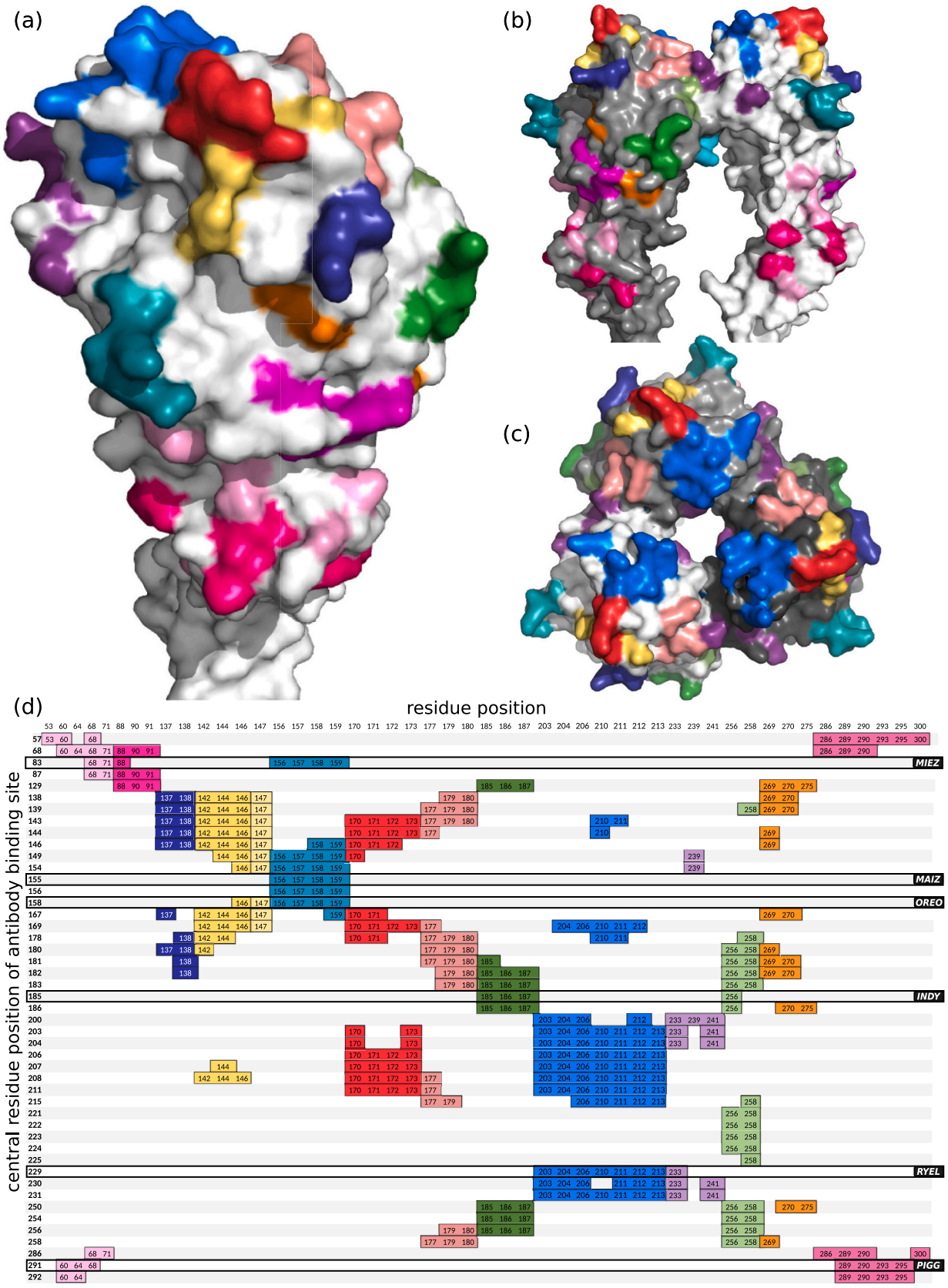

Fig. 3. The relationships between variable regions (VR) on the head region of HA. Shown on (a) a single monomer, (b) at the junction of two monomers, and (c) on the trimer as viewed from above (colours correspond to Table S1). Each line in (d) indicates the variable residues contained within a set of hypothetical antibody-binding sites of 800 Å and accessibility 10. Variable regions (VRs) are indicated by different colours. Seven ELVs are identified in (d) (marked out by the bold outlines on the right): MIEZ (residues 68, 71, 88, 156–159), MAIZ (residues 156–159), OREO (residues 146, 147, 156–159), INDY (residues 138, 14, 144, 170, 171, 177, 179, 180, 210, 211, 258), RYEL (residues 203, 204, 206, 210–213), PIGG (residues 60, 64, 68, 289, 290, 293, 295).

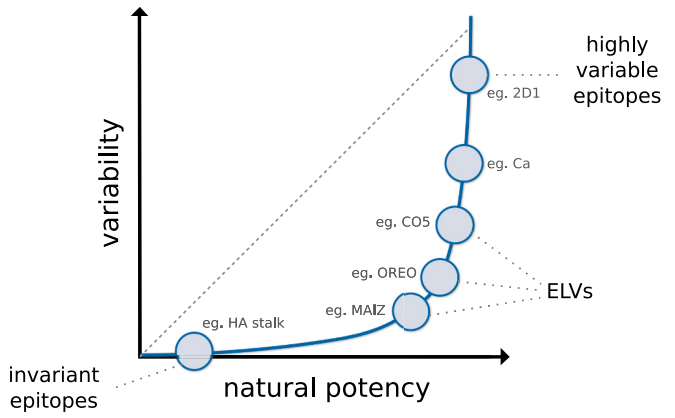

Fig. 4. Schematic representation of the relationship between epitope variability and potency within our framework. The dashed line represents a scenario where potency increases linearly with variability.

such puzzling observations as "very little drift occurred in the Ca site despite the relatively high virus-neutralizing of anti-Ca antibodies in vitro" (Gerhard et al., 1981), by demonstrating that it is the architecture of that region that determines its variability rather than level of selection pressure. Indeed, it is a common misconception that the absence of variability indicates that a site is under lower immune pressure; there are structural constraints that primarily dictate whether immune evasion is possible (cf. for the measles virus, viral escape from neutralisation is impossible as it leads to loss of receptor-binding activity (Tahara et al., 2013)).

An ELV under strong immune selection will cycle through its various conformations, as shown in Fig. 1. This is manifest in patterns of cross-neutralisation as observed by us (Thompson et al., 2018) between A/Solomon Islands/2006, PR8 and A/WSN/1933 which all share red OREO, as well as cross-protection in mice from lethal PR8 challenge upon vaccination with the 2006 red OREO variant. Vaccinating mice with a 1918-like strain has been shown to confer protection against lethal challenge with the 2009 H1N1 strain of comparable levels to homologous inoculation (Manicassamy et al., 2010); both 2009 and 1918 contain the blue OREO variant, as do the two swine viruses (Sw/30 and NJ/30) used in the same experiment which also gave high levels of protection. A range of other historically distributed human viruses (Wei/43, USSR/77, TX/91, Bris/07) which do not contain blue OREO showed more rapid weight loss and slower recovery, indicating that (i) antibodies against OREO play an important role in protection and (ii) there are other shared ELVs (as predicted by the model [12]and other experimental studies (Recker et al., 2007; Linderman et al., 2014)) which also contribute to protection. The re-emergence of blue OREO in the 2009 pandemic strain is also suggested by the high prevalence of neutralising antibodies to it in older individuals who were likely to have been naturally exposed to the 1918 strain (Hancock et al., 2009; Miller et al., 2010). The newly reported H1N2 strain from November 2023 (Cogdale et al., 2024) contains an OREO epitope that is effectively identical to the variant circulating between 1989 and 1996 and also between 1935 and 42 (Fig. S1).

It has recently been shown (Van Reeth et al., 2023) that sequential vaccination with a set of H1N1 strains is able to induce a wide spectrum of neutralising antibodies and protection against challenge with heterologous strains in swine. An inspection of the HA sequences used in this study reveals that all VR2 variants identified in the human H1N1 historical data were covered, thereby suggesting that at least part of the protection derived from neutralising antibodies targeting an ELV containing VR2. Sequential infection of ferrets, both with historical and contemporary H1N1 strains has also been shown to confer some protection against weight loss upon challenge with 2009 H1N1, as well as to significantly reduce ability to transmit to naïve ferrets (Carter et al., 2013). These experiments suggest that a straightforward option for exploiting the existence of ELVs is to vaccinate individuals with a cocktail of relevant strains. However, we suggest that using a prime-boost strategy to focus the response on a particular ELV (Thompson et al., 2018) may be more likely to yield robust and durable protection.

In summary, we have identified a set of novel ELVs on the head of haemagglutinin which likely play a critical role in structuring the evolutionary dynamics of H1 influenza and expand the set of options for delivering universal protection against influenza by focusing the immune response on these epitopes through vaccination. An advantage of targetting ELVs is that it exploits natural immunity rather than artificially stimulating responses that are either not naturally protective or commonly produced during natural infection. Alternative approaches that computationally engineer artificial vaccine constructs (Carter et al., 2016; Dzimianski et al., 2023) may elicit broadly cross-reactive antibodies and may eventually provide universal coverage against influenza, but we believe that it will be far more effective to achieve broad and durable protection by targeting naturally protective epitopes of limited variability on the head of the influenza haemagglutinin surface antigen.

4. Materials and methods

4.1. Identification of antibody binding sites (ABS)

We used the publicly available structures (at www.rcsb.org): 1RUZ (A / South Carolina / 1 / 1918, chains H, J, L), IRU7 (A / Puerto Rico / 8 / 1934, chains A, C, E, G, I, K), 6CF7 (A / Solomon Islands / 3 / 2006, chain A) and 3LZG (A / California / 04 / 2009, chains A, C, E, G, I, K) to define antibody binding sites (ABS). The structural bioinformatic pipeline included 3 steps: (1) quantifying accessibility of amino acid residues (AAR), (2) defining theoretical ABS, (3) aggregating results across structures and their chains. For step (1), the accessibility of AAR on H1N1 HA (for all structures and chains independently) was quantified using the Solvent Accessible Surface Area (SASA) C library (v2.0.3, www.freesasa.github.io). In step (2), for every AAR on H1N1 HA (for all structures and chains independently) an spherical area centred at the AAR (ABS pin) with specific radius (in Å) was considered and all AAR within the area were identified (using as references the positions of each AAR's alpha-carbon). Finally, in step (3), for each combination of considered AAR accessibilities (1,10,30) and spherical radiuses (500, 800, 1000) (Fig. S2), AARs belonging to ABSs were identified if present in at least one chain of any structure (Fig. S3).

4.2. Mapping variable regions (VR) onto the HA head

We defined variable regions as collections of variable residues in close proximity both within sequence and structure. For the latter, a range of crystal structures were used (as presented in Figs. 2 and 3), including those mentioned above as well as 6ONA (A / Hickox / 1940), 7JPD (A / Fort Monmouth / 1 / 1947), 6MYA (A / Almaty / 32 / 1998), 6 N41 (A / Netherlands / 002P1 / 1951), 4M4Y (A / California / 04 / 2009), 6CHX (A / Juliaca / FLU3969 / 2006). In some cases, structures deposited in the PDB contained truncated amino acids and these were replaced with complete residues in COOT (Crystallographic Object-Oriented Toolkit). Where crystal structures were not available, AlphaFold2 models were used. Analysis included the entire head from C59 to C292 for H1N1 viruses. Residue variability was assessed by analysing a panel of HA sequences representing the most abundant strain in each year from 1918-present (as presented in Table S1). Sequences were obtained from Genbank for the period 1918 to 2015 as described in (Thompson et al., 2018), and de novo from Genbank for the period 2016 to 2024 following the same approach. Variable residues were defined as those that exhibited change in charge and/or polarity.

4.3. Defining epitopes of limited variability (ELVs)

ELVs can be identified by combining the outputs of the ABS and VR analyses (described above) under the heuristic of minimizing the number of VRs included within the ABS footprint (examples in Fig. 3).

CRediT authorship contribution statement

José Lourenço: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Hany Zinad:** Writing – review & editing, Validation, Methodology, Investigation, Data curation. **James Kempton:** Writing – review & editing, Validation, Formal analysis, Data curation. **Sunetra Gupta:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Sunetra Gupta, José Lourenço and Hany Zinad report financial support was provided by Blue Water Vaccines. Sunetra Gupta has patent #US 11,123,422 B2, PCT/GB2017/052510 licensed to Sunetra Gupta. Sunetra Gupta and José Lourenço declare they are shareholders of Orto Bio. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data is available in online repositories or in supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2026.105926.

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