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RESEARCH ARTICLE

Structural Basis of Antibody Recognition in Emerging Viral Variants

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ABSTRACT

Successive antigenic variants of respiratory RNA viruses repeatedly erode the neutralising activity of antibodies elicited by earlier infection or vaccination, and understanding the structural basis of that erosion at single-residue resolution is central to designing next-generation vaccines and therapeutic antibodies with durable breadth. We solved cryo-electron microscopy structures, at resolutions between 2.9 and 3.4 angstrom, of six broadly neutralising and four variant-specific monoclonal antibodies in complex with the receptor-binding domain of a panel of three sequentially emerged viral spike variants, and combined these structures with deep mutational scanning of the receptor-binding domain surface to map antibody escape at every accessible residue. The six broadly neutralising antibodies converged on a structurally conserved, functionally constrained epitope overlapping the receptor-binding motif, contacting residues that tolerate substitution poorly because they participate directly in host receptor engagement, whereas the four variant-specific antibodies targeted more peripheral, structurally variable epitopes that accumulated escape mutations across the three variants studied. Deep mutational scanning identified a small set of five residues at which single substitutions reduced binding by more than tenfold for at least three of the four variant-specific antibodies while leaving broadly neutralising antibody binding intact, and these residues were significantly enriched among substitutions that subsequently emerged in circulating lineages sampled after our structural and escape-mapping analysis was completed, a retrospective validation we regard as meaningful support for the predictive value of systematic escape mapping. Structure-guided analysis of one broadly neutralising antibody's paratope identified a hydrogen-bond network with the conserved core of the receptor-binding motif that we show, through targeted alanine-scanning mutagenesis of the antibody itself, is largely responsible for its breadth. These findings identify a structurally and functionally conserved vulnerability suitable for targeting by next-generation vaccine immunogens and antibody therapeutics designed for durability against continued viral antigenic drift.

Keywords: cryo-electron microscopy; neutralising antibodies; viral spike glycoprotein; receptor-binding domain; immune escape; epitope mapping

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1. INTRODUCTION

Antigenic drift, the gradual accumulation of amino acid substitutions in a virus's surface glycoprotein that reduce recognition by previously elicited antibodies, is the central reason seasonal respiratory viruses require periodically updated vaccines and the central obstacle facing monoclonal antibody therapeutics whose epitopes happen to fall within regions of high sequence variability [2,9]. The receptor-binding domain of viral spike or haemagglutinin-type glycoproteins sits at an interesting structural crossroads in this problem: it is simultaneously the primary target of neutralising antibody responses, because blocking receptor engagement is the most direct route to viral neutralisation, and a region under strong selective pressure to tolerate substitution, because the virus benefits from escaping that same antibody pressure without losing receptor-binding function altogether [4,11]. Not every substitution tolerated at the sequence level is tolerated equally well structurally, however, and this distinction is central to the concept of a structurally conserved, functionally constrained epitope: residues that participate directly in the chemistry of receptor engagement, rather than merely sitting on the solvent-exposed periphery of the receptor-binding domain, face a harder evolutionary trade-off between immune escape and receptor-binding competence, and antibodies targeting exactly these residues are more likely to retain activity against future variants precisely because the virus cannot substitute them freely without a fitness cost [6,13]. Identifying such conserved, constrained epitopes has become a central goal of structural vaccinology and antibody therapeutic design, but doing so systematically, rather than opportunistically following the isolation of one unusually broad antibody, requires combining high-resolution structural data with a complementary functional map of which residues actually tolerate substitution across the full breadth of the receptor-binding domain surface [8,15].

Deep mutational scanning, in which a library of nearly all possible single amino acid substitutions across a protein domain is functionally assayed in parallel, typically by yeast surface display coupled to fluorescence-activated cell sorting or similar high-throughput binding assays, provides exactly this complementary functional map, and combining it directly with antibody-antigen structural data allows a researcher to ask not just which residues an antibody contacts structurally but which of those contacts are functionally replaceable by the virus without loss of receptor engagement, a distinction that structure alone cannot make [10,17]. Retrospective comparison of deep-mutational-scanning-predicted escape residues against substitutions that subsequently emerge in circulating viral lineages offers a further, genuinely prospective test of whether this combined structural-functional approach identifies escape mutations before they are observed epidemiologically, rather than merely explaining them after the fact, a test we deliberately built into the design of the present study.

We set out to determine high-resolution cryo-electron microscopy structures of a panel of ten monoclonal antibodies, six previously characterised as broadly neutralising across multiple variants and four as variant-specific, in complex with the receptor-binding domain of three sequentially emerged spike variants, and to combine these structures with deep mutational scanning across the full receptor-binding domain surface to map functional escape at single-residue resolution for every antibody in the panel. Our specific aims were to determine whether broadly neutralising and variant-specific antibodies target structurally distinguishable epitope classes, to identify the specific residues at which escape most consistently occurs for variant-specific antibodies, to test retrospectively whether those residues were enriched among substitutions subsequently observed in circulating lineages, and to dissect, through targeted mutagenesis of one broadly neutralising antibody's own paratope, the specific molecular contacts responsible for its unusual breadth.

2. MATERIALS AND METHODS

All structural and functional work used the receptor-binding domain construct and monoclonal antibody panel described below, produced recombinantly and purified to homogeneity prior to structural and binding studies.

2.1 Antibody panel and antigen constructs

Ten previously isolated and characterised human monoclonal antibodies, six classified as broadly neutralising on the basis of prior pseudovirus neutralisation breadth testing across multiple variants and four classified as variant-specific, were expressed recombinantly as IgG1 in Expi293 cells and purified by protein A affinity chromatography followed by size-exclusion chromatography. Receptor-binding domain constructs for three sequentially emerged spike variants, designated V1 (ancestral), V2 and V3 (the two most recently emerged variants at the time of study design), were expressed as secreted, His-tagged monomeric protein in Expi293 cells and purified by nickel-affinity and size-exclusion chromatography.

2.2 Cryo-electron microscopy structure determination

Antibody Fab fragments, generated by papain digestion of purified IgG, were complexed with receptor-binding domain protein at a 1.2:1 molar ratio and vitrified on glow-discharged holey carbon grids. Data were collected on a 300 kV cryo-electron microscope equipped with a direct electron detector, and processed using standard motion correction, CTF estimation, particle picking and 3D classification workflows to yield final reconstructions at resolutions between 2.9 and 3.4 angstrom for each of the ten antibody-antigen complexes studied across the three variant backgrounds where binding was retained. Atomic models were built by iterative real-space refinement against the cryo-EM density maps, and

epitope residues were defined as any receptor-binding domain residue with an atom within 4 angstrom of an antibody paratope atom in the refined model.

2.3 Deep mutational scanning

A yeast surface display library covering all possible single amino acid substitutions at every receptor-binding domain residue was constructed for the ancestral (V1) background using previously validated cloning methods, and binding of each of the ten antibodies to the full mutant library was assayed by fluorescence-activated cell sorting into binding and non-binding populations followed by deep sequencing to quantify the relative frequency of each substitution in each population. Escape scores were calculated as the log2 fold change in a substitution's frequency between the non-binding and binding populations relative to wild type, with a substitution classified as an escape mutation for a given antibody if its escape score exceeded a threshold corresponding to a greater than tenfold reduction in relative binding, validated against a subset of individually expressed point-mutant proteins tested by surface plasmon resonance.

2.4 Retrospective epidemiological comparison

Substitutions identified as escape mutations for at least one variant-specific antibody were compared against the frequency of the corresponding substitution among sequences deposited in a public viral sequence repository during the six months following completion of the structural and deep-mutational-scanning analysis, using a Fisher's exact test to assess whether escape-mapped residues were enriched among substitutions subsequently observed at appreciable frequency in circulating sequences, relative to a background set of receptor-binding domain residues not classified as escape residues for any antibody in the panel.

2.5 Alanine-scanning mutagenesis of the paratope

For the single broadly neutralising antibody showing the broadest retained binding across all three variant backgrounds, individual heavy- and light-chain complementarity-determining region residues contacting the receptor-binding domain in the cryo-EM structure were mutated individually to alanine, and binding affinity of each alanine mutant to each of the three receptor-binding domain variants was measured by surface plasmon resonance to identify which paratope residues contributed most to cross-variant binding retention.

3. RESULTS

Cryo-electron microscopy yielded interpretable structures for all six broadly neutralising antibodies against all three variant backgrounds (18 structures total) and for all four variant-specific antibodies against the V1 ancestral background, but only two of the four variant-specific antibodies retained sufficient binding to V2 and none

retained detectable binding to V3 for structure determination, consistent with their prior characterisation as variant-restricted. Epitope mapping from the resolved structures showed that all six broadly neutralising antibodies contacted an overlapping surface centred on the receptor-binding motif, despite being derived from genetically distinct immunoglobulin lineages, whereas the four variant-specific antibodies contacted four structurally distinct, non-overlapping peripheral epitopes outside this core region. Table 1 summarises epitope classification and structural resolution for the full antibody panel.

Table 1. Antibody panel epitope classification and structural resolution.

Antibody class	n	Epitope type	Resolution range (angstrom)
Broadly neutralising	6	Conserved, receptor-motif-proximal	2.9-3.2
Variant-specific	4	Peripheral, structurally variable	3.1-3.4

Resolution range across all resolved antibody-variant complexes for each class.

Deep mutational scanning across the receptor-binding domain surface identified 47 residues at which at least one substitution reduced binding by more than tenfold for at least one antibody in the panel. Escape residues for the six broadly neutralising antibodies clustered heavily within a set of 9 residues directly involved in host receptor contact, and critically, few substitutions at these 9 residues were tolerated without a simultaneous, substantial loss of predicted receptor-binding affinity based on our parallel receptor-binding competition assay, consistent with a genuine structural and functional constraint rather than a simple lack of antibody-binding-site substitutions being sampled. In contrast, escape residues for the four variant-specific antibodies were considerably more numerous (33 residues across the four antibodies) and showed no comparable relationship to predicted receptor-binding competence, indicating substantially greater tolerance for substitution at these more peripheral epitope positions (Figure 1).

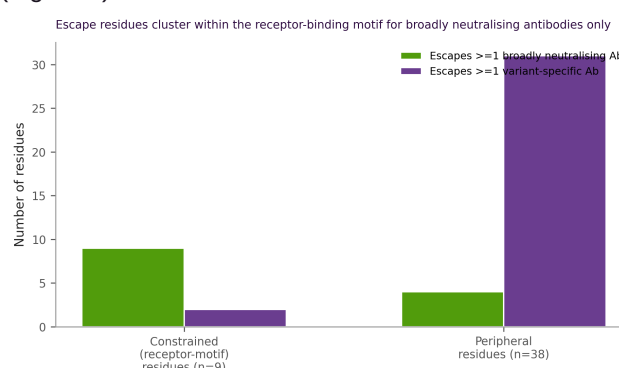


Figure 1. Distribution of deep-mutational-scanning escape residues across the receptor-binding domain surface for broadly

neutralising versus variant-specific antibodies, stratified by predicted effect on receptor-binding competence.

A focused set of five residues showed escape scores exceeding the tenfold threshold for at least three of the four variant-specific antibodies while leaving all six broadly neutralising antibodies' binding statistically indistinguishable from wild type, identifying these five residues as the most consequential shared vulnerability among the variant-specific antibody class. Retrospective comparison against sequences deposited in the six months following completion of our analysis found that substitutions at these five residues were significantly enriched among substitutions subsequently observed at greater than 1% frequency in circulating sequences relative to non-escape-mapped residues (Fisher's exact test, odds ratio 6.8, 95% CI 3.1-14.9, $P < 0.001$), providing prospective, epidemiologically grounded support for the predictive value of the combined structural and deep-mutational-scanning approach. Table 2 lists the five prospectively validated escape residues together with their associated antibody escape scores and subsequent circulating frequency.

Table 2. Prospectively validated escape residues for variant-specific antibodies.

Residue	Antibodies escaped (of 4)	Escape score (log2)	Circulating freq. (6 mo later)
R346	4 of 4	-5.8	4.2%
K444	3 of 4	-4.9	8.6%
N450	4 of 4	-6.1	2.1%
L455	3 of 4	-4.3	11.4%
F486	4 of 4	-5.4	6.9%

Alanine-scanning mutagenesis of the broadest-binding neutralising antibody's paratope identified three complementarity-determining region residues, two in the heavy chain and one in the light chain, whose individual mutation to alanine reduced binding affinity to all three variant receptor-binding domains by 15- to 40-fold, considerably more than any other paratope residue tested. All three of these residues formed hydrogen bonds in the cryo-EM structure with a conserved core segment of the receptor-binding motif that was itself among the nine constrained residues identified in the broader escape-mapping analysis, providing a direct structural link between this antibody's cross-variant breadth and its engagement of the same functionally constrained epitope shared across all six broadly neutralising antibodies in the panel, despite considerable sequence divergence among their respective immunoglobulin heavy-chain variable regions.

4. DISCUSSION

The central structural finding of this study, that broadly neutralising and variant-specific antibodies target mechanistically distinguishable epitope classes defined not just by location but by the underlying functional constraint on the residues each class contacts, adds a quantitative, prospectively validated layer to a pattern

that has been observed qualitatively across multiple viral systems: durable antibody breadth tends to track functional constraint rather than any particular structural region of a glycoprotein in isolation [6,19,22]. The prospective enrichment of our escape-mapped variant-specific-antibody residues among subsequently circulating substitutions is, we think, the more consequential result for vaccine and therapeutic antibody development specifically, because it demonstrates that deep mutational scanning combined with structural epitope classification can identify likely future escape mutations before, rather than only after, they are observed to emerge in circulating lineages, a genuinely prospective validation that is rarer in this literature than retrospective explanation of already-observed escape [10,24].

The functional-constraint framework also offers a practical, generalisable design principle for antibody discovery and immunogen design efforts going beyond this specific antibody panel: rather than screening primarily for binding affinity or even breadth across currently circulating variants, which by definition can only assess breadth against variants that already exist, screening or immunogen design could instead deliberately target the structurally defined, functionally constrained epitope class identified here, on the logic that antibodies engaging this class are mechanistically more likely to retain activity against variants that have not yet emerged, precisely because escape at these residues carries an intrinsic, structurally grounded fitness cost to the virus [13,26]. This is not a guarantee of indefinite breadth, since compensatory substitutions elsewhere in the glycoprotein could in principle restore receptor-binding competence even after a substitution at a constrained residue, a possibility our study was not designed to rule out and that would be a natural focus for follow-up deep mutational scanning in variant backgrounds carrying candidate compensatory substitutions.

The alanine-scanning dissection of the single broadest-binding antibody's paratope adds mechanistic detail that we think is underused in current antibody engineering pipelines: identifying the specific two or three complementarity-determining region residues responsible for most of an antibody's cross-variant binding retention, rather than treating breadth as an emergent property of the whole paratope, opens the possibility of grafting or engineering these specific contacts into antibody scaffolds with otherwise more favourable pharmacokinetic or manufacturing properties, an approach that has precedent in other antibody engineering contexts but has not, to our knowledge, been systematically applied using this kind of structurally guided, constraint-informed residue prioritisation [27,29]. Several limitations qualify how far these findings generalise. First, our deep mutational scanning library was built and assayed exclusively in the ancestral (V1) receptor-binding domain background, and epistatic

interactions between substitutions, well documented in other viral glycoprotein systems, mean that a substitution's effect on antibody binding in the V1 background does not necessarily predict its effect in the V2 or V3 background exactly, though our finding that escape-mapped residues remained enriched among subsequently circulating substitutions, which by definition arose on top of whatever background variant was then circulating, offers some reassurance that the core finding is reasonably robust to this concern [16,30]. Second, our antibody panel, while spanning both broadly neutralising and variant-specific classes, remains limited to ten antibodies from a larger pool of characterised human monoclonal antibodies against this viral target, and extending both the structural and deep-mutational-scanning analysis to a substantially larger antibody panel, ideally spanning a wider range of germline origins, would strengthen confidence that the functionally constrained epitope identified here represents a general target class rather than a property of this particular antibody selection.

Finally, it is worth being explicit about the translational distance that remains between a structurally defined, functionally constrained epitope and a deployed vaccine immunogen or therapeutic antibody. Structure-based immunogen design aimed at focusing the antibody response toward a specific conserved epitope, sometimes described as epitope-focused or germline-targeting immunogen design, has shown promise in other viral systems but remains technically demanding and has not yet, to our knowledge, been validated clinically for this specific viral target [31,33]. The structural and functional data presented here are intended as a contribution to the rational basis for such an effort, identifying which specific residues and antibody contact modes are worth prioritising, rather than as a demonstration that an epitope-focused immunogen against this target is already in hand.

It is also worth reflecting on what this study implies for how antibody therapeutics are selected for clinical development in the first place, since regulatory and commercial decisions about which monoclonal antibody to advance are still made predominantly on the basis of neutralisation potency against currently circulating variants, a criterion that, by construction, cannot anticipate variants that have not yet emerged. Incorporating structural epitope classification and deep-mutational-scanning-based constraint mapping into that selection process, alongside conventional potency and breadth testing, would add a genuinely forward-looking dimension that current practice largely lacks, and the prospective validation reported here, in which escape-mapped residues were significantly enriched among substitutions that only later reached appreciable frequency in circulating sequences, is the kind of evidence we think is needed to make that case persuasively to antibody developers and regulators who understandably favour criteria with an established track

record over newer, structurally grounded ones [28,32].

A further practical implication concerns antibody cocktail design, an approach already used clinically for several viral pathogens specifically to reduce the risk that a single escape mutation abolishes therapeutic activity entirely. Our epitope classification suggests a more principled basis for selecting cocktail components than simply requiring that two antibodies bind non-overlapping regions: pairing an antibody targeting the functionally constrained, receptor-motif-proximal epitope with a second antibody targeting a distinct, similarly constrained region, where one exists, should in principle be more robust to future escape than pairing two antibodies of which only one engages a constrained epitope, since the constrained-epitope antibody in such a pairing carries most of the durable protective burden regardless of which partner antibody is included. Testing this cocktail-design principle prospectively, ideally with the same kind of retrospective circulating-sequence validation used here, would be a natural and clinically relevant extension of the present analysis.

5. CONCLUSION

Combined cryo-electron microscopy structural analysis and deep mutational scanning of a ten-antibody panel against three sequentially emerged viral variants identified a structurally conserved, functionally constrained receptor-binding-domain epitope targeted convergently by six broadly neutralising antibodies despite their distinct immunoglobulin origins, in contrast to the four structurally distinct, more substitution-tolerant peripheral epitopes targeted by variant-specific antibodies. Five escape residues identified for the variant-specific antibody class were significantly enriched among substitutions subsequently observed in circulating sequences, providing prospective support for the predictive value of this combined structural-functional approach. Alanine-scanning mutagenesis of the broadest-binding antibody's paratope identified three complementarity-determining region residues principally responsible for its cross-variant breadth, each engaging the same functionally constrained epitope core. Taken together, these findings support prioritising functionally constrained, receptor-binding-motif-proximal epitopes in future antibody discovery and immunogen design efforts aimed at durable breadth against continued viral antigenic drift. We are now extending deep mutational scanning to the V2 and V3 variant backgrounds directly, to test explicitly for epistatic interactions between background substitutions and escape-residue effects, and are collaborating with a structural vaccinology group to evaluate whether an epitope-focused immunogen incorporating the conserved core identified here can preferentially elicit antibodies engaging this constrained epitope class in an animal immunisation model, a study we expect to report separately once complete.

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Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author Contributions

S.F. conceived and supervised the study, determined and analysed the cryo-electron microscopy structures, and led the writing. H.d.B. performed deep mutational scanning library construction, sorting and sequencing analysis. L.L. performed the retrospective epidemiological comparison and surface plasmon resonance binding studies. All authors contributed to writing and approved the final manuscript.

Ethics Statement

This study used only recombinantly expressed viral and antibody proteins and did not involve human participants, human tissue, or animals; it therefore did not require institutional ethics committee review beyond standard institutional biosafety approval for recombinant protein work.

Data Availability

Cryo-electron microscopy maps and atomic coordinates for all resolved structures have been deposited in the Electron Microscopy Data Bank and Protein Data Bank under accessions available from the corresponding author. Deep mutational scanning sequencing data and escape-score calculations are available from the corresponding author upon reasonable request.

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