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# Antibody-mediated synergy and interference in the neutralization of SARS-CoV at an epitope cluster on the spike protein

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ABSTRACT

Incomplete neutralization of virus, especially when it occurs in the presence of excess neutralizing antibody, represents a biological phenomenon that impacts greatly on antibody-mediated immune prophylaxis of viral infection and on successful vaccine design. To understand the mechanism by which a virus escapes from antibody-mediated neutralization, we have investigated the interactions of non-neutralizing and neutralizing antibodies at an epitope cluster on the spike protein of severe acute respiratory syndrome coronavirus (SARS-CoV). The epitope cluster was mapped at the C-terminus of the spike protein; it consists of structurally intertwined epitopes recognized by two neutralizing monoclonal antibodies (mAbs), 341C and 540C, and a non-neutralizing mAb, 240C. While mAb 341C binds to a mostly linear epitope composed of residues $^{507}$PAT$^{509}$ and V$^{349}$, mAb 240C binds to an epitope that partially overlaps the former by at least two residues (P$^{507}$ and A$^{508}$). The epitope corresponding to mAb 540C is a conformational one, involving residues L$^{504}$ and N$^{505}$. In neutralization assays, non-neutralizing 240C disrupted virus neutralization by mAb 341C and/or mAb 540C, whereas a combination of mAbs 341C and 540C blocked virus infectivity synergistically. These findings indicate that the epitope cluster on the spike protein may serve as an evolutionarily conserved platform at which a dynamic interplay between neutralizing and non-neutralizing antibodies occurs, thereby determining the outcome of SARS-CoV infection.

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## Introduction

Patients suffering from infection with severe acute respiratory syndrome coronavirus (SARS-CoV) [1] often have appreciable levels of virus-specific antibody [2–5]. This situation is similar to that encountered in other viral diseases such as chronic hepatitis C or HIV, where the infection persists despite the presence of antibodies [6–10]. One of the mechanisms proposed originally by Dulbecco et al. [11] to account for apparent inhibition of virus neutralization was that the serum of infected individuals might contain non-neutralizing antibodies. When combined with the corresponding virus, the non-neutralizing antibody could presumably interfere with the attachment of neutralizing antibody, thereby diminishing its neutralizing activity. Massey and Schochetman subsequently showed that non-neutralizing antibody binds to virus and sterically blocks the binding of neutralizing antibodies [12]. Recently, we found two Epitopes I and II, within a short peptide between the hypervariable regions I and II of the hepatitis C virus E2 protein. Epitope I, but not Epitope II, was implicated in virus neutralization. The binding of a non-neutralizing antibody to Epitope II disrupted virus neutralization mediated by antibody binding at Epitope I [9,13].

Here, we have investigated whether or not a similar occurrence, namely the presence of non-neutralizing antibody along with neutralizing antibody, could account for the observation that SARS-CoV survives despite a vigorous antibody response by the host. We have characterized three previously isolated monoclonal antibodies (mAbs) that recognize the spike protein of SARS-CoV between residues 491 and 510 [14]. We found that the two neutralizing mAbs, 341C and 540C, could act synergistically to inhibit SARS-CoV infection *in vitro*, while the non-neutralizing antibody, 240C, disrupted the neutralizing activity of both 341C and 540C. These findings suggest that this epitope cluster may provide a viral escape mechanism whereby the neutralization of virus is thwarted by an interfering, non-neutralizing antibody.

## Materials and methods

*Monoclonal antibodies.* Monoclonal anti-SARS-CoV antibodies, 240C, 341C and 540C, were obtained from the Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH. mAbs 341C and 534C could neutralize SARS-CoV infection of Vero

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E6 cells, while mAb 240C did not. The epitopes of these mAbs were located within residues 491–510 on the spike protein. The mAb 540C used in the present study is similar to 534C as described previously [14].

*Virus stock and micro-neutralization assay.* Vero E6 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. The Urbani strain of SARS-CoV was plaque-purified, grown to stock titers in Vero E6 cells, purified by polyethylene glycol (PEG) precipitation as described previously [15], and frozen at −70 °C until use.

A micro-neutralization assay was performed as previously described [16]. The micro-neutralization titer of test antibody was the highest dilution that showed inhibition in all triplicate wells. Controls were included for each assay performed and included back titration, inclusion of positive control antibody (i.e., serum from a convalescent SARS patient) and an isotype monoclonal antibody control. Data obtained from at least three independent experiments were analyzed.

*Peptide synthesis.* All peptides were synthesized by the Core Laboratory of the Center for Biologics Evaluation and Research, Food and Drug Administration, with an Applied Biosystems (Foster City, CA) Model 433A Peptide Synthesizer by using standard FastMoc chemistry [17].

*ELISA.* Streptavidin-coated 96-well plates were used for ELISA according to the manufacturer's instructions (Pierce, Rockford, IL). Briefly, biotinylated peptides (200 ng/well) were added to streptavidin-coated wells and blocked with Blocking Buffer for 1 h at 37 °C. After washings with PBS with 0.05% Tween 20 (PBS-T), primary antibody was added to the wells and incubated for 45 min at 37 °C. After removal of unbound antibodies by washing with PBS-T, a goat anti-mouse peroxidase-conjugated IgG (KPL, Gaithersburg, MD) at 1:5000 dilution was added to the wells and incubated for 30 min at 37 °C. After washings, tetramethylbenzidine substrate (Pierce) was added and the plates were incubated at room temperature in the dark for 10 min. The reaction was terminated by adding 4 N sulfuric acid, and absorbance at 450 nm was measured (Optimax; Molecular Devices, Palo Alto, CA).

*Phage display.* Selection of peptides from random peptide phage display libraries (New England Biolabs, Beverly, MA) was described previously [18]. Briefly, $10^{10}$ phages were incubated with individual antibody/protein G mixtures for 20 min at room temperature. After eight washings with 0.05 M Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl and 0.05% Tween 20, the phages were eluted from the complex with 0.1 M HCl for 8 min at room temperature and neutralized with 1 M Tris–HCl (pH 9.0). The eluted phages were then amplified in the host strain ER2738. After three additional rounds of selection of amplified phages, DNA from each single-phage plaque was sequenced, and the corresponding peptide sequence was then deduced from the DNA sequence.

*Structural analysis.* The structural analysis was performed by using the coordinates of the SARS-CoV receptor-binding domains (RBD), i.e., PDB IDs 2DD8, 2AJF, 2GHW and 2GHV [19–22]. Among these structures, PDB ID 2DD8 has a complete assignment for the C-terminal region of RBD encompassing residues 491–510 for the antibody binding. The structure was visualized and manipulated by using the program PyMOL 0.99rc6 [23].

*Statistical analysis.* JMP v.5.0 software (SAS Institute, Cary, NC) was used for analyzing data. Pair-wise comparisons of the means between peptide mutations were performed by using the Student's *t*-test where $p < 0.05$ was considered statistically significant. For an overall comparison of means, the Tukey–Kramer HSD test was used. Statistical significance was set at $\alpha = 0.05$. A positive test value generated between two means is indicative of a significant difference.

## Results

*Identification of a cluster of epitopes recognized by mAbs 240C, 341C and 540C at the C-terminus of the spike protein*

We attempted to identify the residues within fragment 491–510 that are critical for antibody recognition. A peptide encompassing residues 491–510 was chemically synthesized. ELISA analysis showed that only mAb 341C was capable of recognizing the synthetic peptide, whereas mAbs 240C and 540C were unable to bind to it (Fig. 1A). These results suggested that the epitope corresponding to mAb 341C was likely to be a linear epitope, consisting of residues within peptide 491–510.

To characterize the epitope for mAb 341C antibody, we made use of previously published structural information about the spike protein [19] to guide the synthesis of a set of peptides that contained specific mutations (Fig. 1B). Replacement of $N^{505}A^{506}$ with RR in the peptide spanning residues 491–510 had no detectable effect on the binding of mAb 341C, suggesting that neither $N^{505}$ nor $A^{506}$ was involved in the epitope for mAb 341C. However, a single mutation of $P^{507}$ to R resulted in significant loss of binding by mAb 341C ($p < 0.05$) (Fig. 1C). Similarly, a reduction of binding was observed following the replacement of $T^{509}V^{510}$ with RR ($p < 0.05$) (Fig. 1C). The results indicated that residue $P^{507}$, as well as $T^{509}$ and/or $V^{510}$ within peptide 491–510, is important for mAb 341C

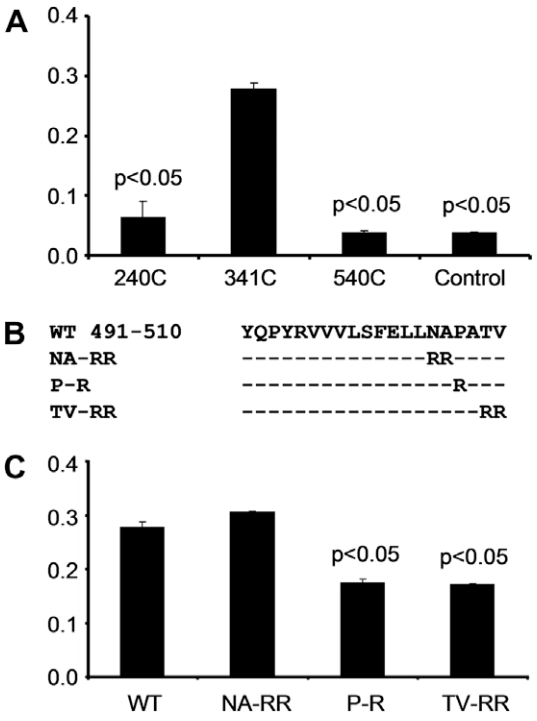


**Fig. 1.** Epitope mapping. (A) Epitope mapping by ELISA. One hundred nanograms biotin-labeled peptide WT 491–510 was added to streptavidin-coated 96-well plates in an ELISA. The *X*-axis indicates different mouse monoclonal antibodies and a control. The *Y*-axis indicates absorbance at 450 nm in ELISA. Absorbance of mAb 341C is compared with that of mAb 240C, mAb 540C and control antibody. *t* test results are shown above each column. $p < 0.05$ is considered significant. (B) Peptide 491–510 mutants. Amino acid sequences encompassing residues 491–510 (WT) and its mutations (NA > RR, P > R and TV > RR) are indicated. (C) ELISA of peptide 491–510 mutants. Biotin-labeled peptide 491–510 (100 ng/well), defined as WT, and its mutants, NA > RR, P > R and TV > RR, respectively, were added to streptavidin-coated 96-well plates. MAb 341C at 1:100 dilution that reacted with WT peptide was used as the probe The *X*-axis indicates peptides used in the assay. The *Y*-axis indicates absorbance at 450 nm, representing specific binding of mAb 341C to each individual peptide. *t* test results of WT and mutants comparison are shown above each column. $p < 0.05$ is considered significant.

recognition. Indeed, by inserting residues $^{505}$NAPATV$^{510}$ into a synthetic peptide derived from the HCV E2 protein, we confirmed that $^{505}$NAPATV$^{510}$ alone could be recognized by mAb 341C [9].

The inability of mAbs 240C and 540C to bind to the synthetic peptide 491–510 implied that the epitopes might be conformational in nature. To map the epitopes corresponding to mAbs 240C and 540C, random peptide phage display libraries were used. Two commercially available libraries, C7C and 12-mer, were used in the present study. The C7C library displays 7 amino acids flanked by two cysteine residues, permitting the formation of a disulfide bond. In such a configuration, the peptide residues can be presented on a loop structure, allowing them to be recognized by a single antibody. To complement the C7C approach, screening the 12-mer peptide library by an antibody could assist in identifying the core sequence [18], as defined by at least two connected residues within the displayed peptide, highly similar to, if not identical with, the linear sequence of the spike protein. We found that A, P, and V were the key residues present in the selected peptides following the screening of the C7C library with mAb 240C (Fig. 2A). When the 12-mer library was screened, three connected residues (APA) were found as the core sequence (Fig. 2A). As these linear residues were found in peptide 491–510, we were able to determine that the epitope corresponding to 240C was composed of the residues $^{506}$APA$^{508}$ (Fig. 2B).

V$^{510}$ is not exposed on the surface, but rather is buried inside the structure of the spike protein [19], thus making it inaccessible for antibody binding. We then surveyed other valine residues located proximal to the core sequence of $^{506}$APA$^{508}$. We found that, by virtue of the particular folding of the spike protein, V$^{349}$ is situated close to $^{506}$APA$^{508}$. The resulting unique position of V$^{349}$ makes it an ideal structural substitute for the buried side chain of V$^{510}$ (Fig. 3). This finding provided the rationale for including V$^{349}$ in the proposed binding site for mAb 240C.

Whereas residues P, T and L were found to be critical for the binding of mAb 341C by using C7C library (Fig. 2A), PATL was revealed as the core sequence by 12-mer library, which is nearly identical to $^{507}$PATV$^{510}$. We identified that these residues are involved in the binding of mAb 341C (Fig. 2B). Knowing that V$^{510}$ is neither exposed on the surface nor solvent-accessible, we argue that V$^{349}$, rather than V$^{510}$, is a key residue for mAb 341C binding. In addition, we found that P$^{507}$ and A$^{508}$ were the shared residues recognized by both mAbs 240C and 341C. The possibility of competition between these two functionally distinct mAbs was therefore raised.

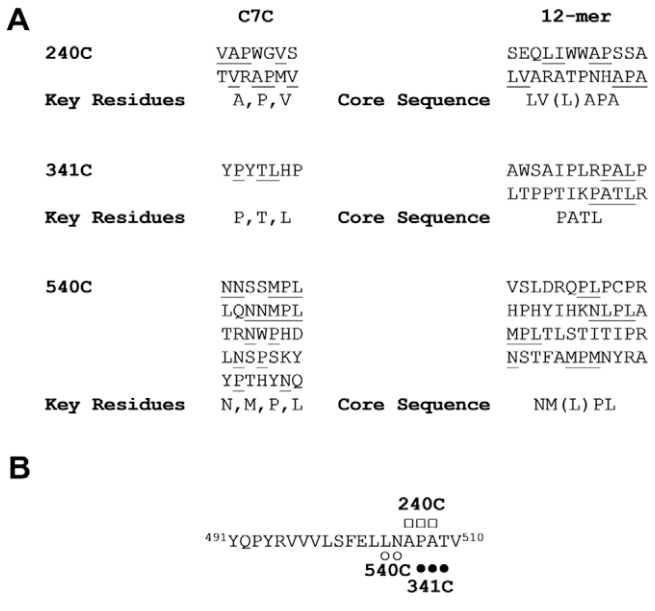


**Fig. 2.** Epitope mapping by random peptide phage display. (A) Key residues and the core sequence consensus for antibody binding. Epitope mapping was conducted by screening two phage display libraries, C7C and 12-mer, with mAbs 240C, 341C and 540C, respectively. (B) Mapping continuous and discontinuous amino acids involved in antibody binding. Residues identified for each antibody are marked with empty squares, closed or open circles.

Similarly, when the C7C library was screened by using mAb 540C, the phage-displayed peptides contained key residues N, M, P, and L. Subsequently, screening of the 12-mer further suggested that residues NM(L)PL were in the core sequence for mAb 540C (Fig. 2A and B). These data indicated that residues L$^{504}$ and N$^{505}$ are the residues for mAb 540C binding. Interestingly, our analysis of the structure of the spike protein revealed that several residues including M$^{416}$, P$^{413}$ and L$^{412}$, i.e., three of those identified by phage display, were exposed on the surface and located on a loop adjacent to the epitopes for mAbs 240C and 341C (Fig. 3C and D). This peculiar configuration may provide an extended interface, in addition to residues L$^{504}$ and N$^{505}$, for the binding of mAb 540C. Further structural studies are needed to confirm this supposition.

### *Structural modeling of the epitope cluster*

The spatial relationships among the three epitopes were analyzed further by structural modeling (Fig. 3). First, we observed that the three identified epitopes form a closely packed cluster and thus are located proximal to each other on the surface of the spike protein (Fig. 3A), yet distal from the ACE2 receptor binding site. The epitope for mAb 240C partially overlaps with that for mAb 341C by at least two amino acids, i.e., P$^{507}$ and A$^{508}$. Presumably, by competing for residues (Fig. 3B), the non-neutralizing antibody mAb 240C could inhibit the binding of neutralizing mAb 341C in an equilibrium-related fashion. Second, the epitope for mAb 240C is also located in close proximity to that recognized by mAb 540C. Binding of mAb 240C may block accessibility to mAb 540C (Fig. 3C) as a large surface area of the antigen is often buried upon antibody binding. Davies and Cohen calculate that the buried area can range from 500 Å$^2$ to more than 800 Å$^2$, encompassing 21–32 amino acid residues, although only 9–20 amino acids (i.e., the epitope) make direct contacts with the antibody [24]. Thus, upon antibody binding, the buried surface area contains more than just the epitope regions that undergo direct interaction with the antibody. It includes also the area adjacent to the epitope regions, plus the area with solvent accessibility blocked by the antibody occupying the surface of the antigen. Third, although the epitopes for mAbs 341C and 540C are located on a single loop in the spike protein, they are spatially separated, thereby providing distinct interfaces for independent antibody binding (Fig. 3D).

### *Antibody-mediated synergy and interference in SARS-CoV neutralization*

The clustering of these epitopes prompted us to examine the functional interaction between their corresponding mAbs measured by virus neutralization assay. Table 1 shows the results obtained from at least three independent neutralization experiments using all the three mAbs independently and/or in combinations. As expected, while mAb 240C did not show any neutralization, mAb 341C and 540C alone neutralized virus infection at titers 2560 and 640, respectively. The neutralization titer rose 4-fold to 10240 when both mAbs 341C and 540C were mixed directly with the virus.

We then asked whether the non-neutralizing mAb 240C could interfere with the neutralizing activities of mAb 341C alone or in combination with mAb 540C (Table 1). As demonstrated by the results from at least three independent neutralization assays, when

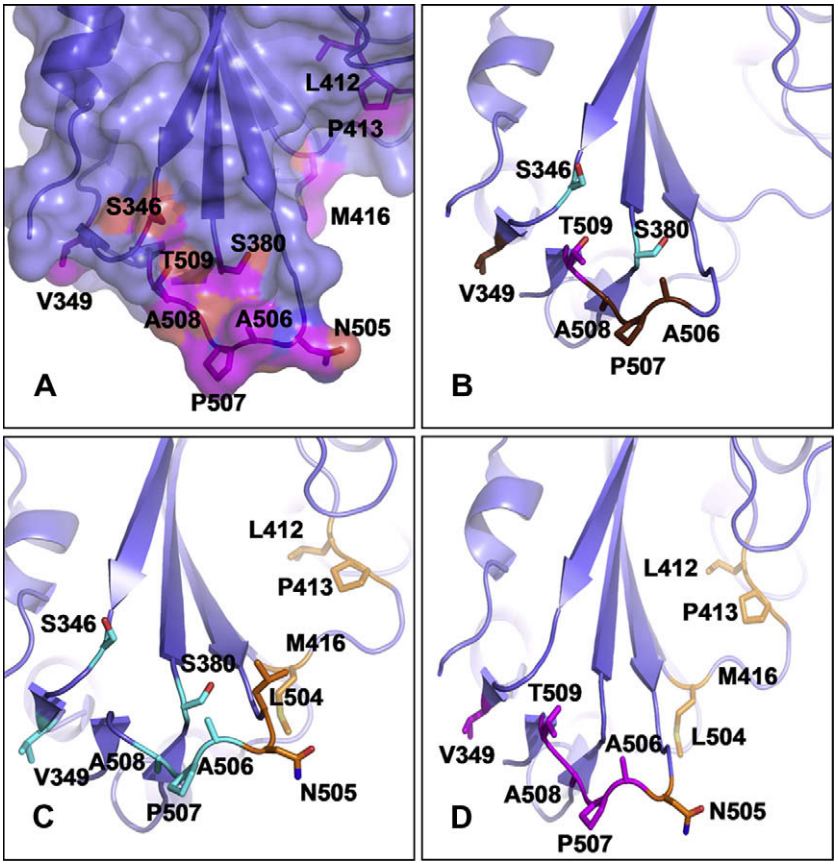


**Fig. 3.** Model for antibody interference and synergy in SARS-CoV infection (A) Exposed amino acids (depicted in purple), participating in antibody binding. (B) Steric interference between mAb 240C and mAb 341C through the proposed mechanisms of residue sharing and spatial occupancy. The residues in cyan are bound by mAb 240C, the residues in magenta are bound by mAb 341C, and the residues in brown are the shared ones for the binding of both antibodies. (C) Steric interference between mAb 240C and mAb 540C through the proposed mechanism of spatial occupancy. The residues in cyan are bound by mAb 240C, while the residues in orange are bound by mAb 540C. (D) Structural and mechanistic model for synergistic virus neutralization between mAb 341C and mAb 540C. The residues in magenta are bound by mAb 341C, while the residues in orange are bound by mAb 540C.

**Table 1**
Synergy and interference of SARS-Cov neutralization in Vero E6 cells.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *Direct Mix*[a] | | | | | | | |
| 240C | + | | | + | + | | + |
| 341C | | + | | + | | + | + |
| 540C | | | + | | + | + | + |
| Titer[b] | <20 | 2560 | 640 | 1280 | 320 | 10240 | 5120 |

| *Stepwise Mix*[a] | | | | |
|---|---|---|---|---|
| 1st mAb | 2nd mAb | | | |
| | 240C | 341C | 540C | Control |
| 240C | – | 1280 | 320 | <20 |
| 341C | 1280 | – | 5120 | 1280 |
| 540C | 320 | 5120 | – | 640 |

[a] mAbs were mixed at a ratio 1:1 simultaneously with virus, and then added to cell culture (Direct Mix); or 1st mAb was mixed with virus and incubated for 30 min before adding 2nd mAb and incubated for additional 30 min. The mixture was then added to cell culture (Stepwise Mix).
[b] Neutralization titers.

both mAbs 240C and 341C were mixed with SARS-CoV, the neutralization titer of mAb 341C was reduced from 2560 to 1280 (Table 1, Direct Mix). Similarly, the presence of 240C also reduced the neutralization titer of mAb 540C from 640 to 320 (Table 1, Direct Mix). The neutralizing activity of the mixture of all three antibodies was lower than that of the two neutralizing antibodies (Table 1, Direct Mix). These results demonstrate that the non-neutralizing mAb 240C can interfere with virus neutralization mediated by mAb 341C and/or mAb 540C.

Finally, we asked whether stepwise mixing of the three mAbs, rather than concomitantly mixing them with the viral stock, would make a difference in neutralizing titers. As shown in Table 1 (Stepwise Mix), there was no significant difference in neutralizing titers when mAbs were added sequentially as compared to adding individual antibodies to the viral inoculum simultaneously. These studies imply that these mAbs have similar binding affinities toward their specific epitopes, and may act in an equilibrium-related manner.

## Discussion

In this study, we have identified a cluster of epitopes on the spike protein of SARS-CoV. Within this cluster an interplay occurs among non-neutralizing and neutralizing antibodies. First, the antibody-specific epitopes are spatially close to each other. This arrangement creates a unique platform for competition. One of possible mechanisms is that the steric block by non-neutralizing antibodies reduces the amount of virion-bound neutralizing antibody on the spike protein to a suboptimal level of occupancy that can disable neutralization, as concluded by Klasse and co-workers from their studies of human and animal viruses [25,26]. Such condition has been reported for West Nile Virus that antibody-mediated neutralization can be achieved only at relatively high concentrations of virion-bound antibody, whereas an enhancement of infection is observed at lower concentrations through conformational triggering of entry functions or through complement- or Fc receptor-dependent mechanisms [27].

Second, although the epitopes of the neutralizing mAbs 341C and 540C are situated spatially close, partly sharing a single loop structure, they represent two separate interfaces for antibody binding. The synergistic neutralization we observed in our mixing experiments may thus be understood as that these two antibodies are able to bind to their respective epitopes without competition.

Third, the residues in the two neutralization epitopes for mAbs 341C and 540C are not directly involved in binding the ACE2 receptor [28]. This finding is not unprecedented; antibodies such as those specific for influenza hemagglutinin neutralize by binding outside the virus receptor binding site [29]. In addition, alternative or auxiliary receptors for SARS-CoV could exist [30–32]. Further study on whether mAb 341C or mAb 540C sufficiently blocks other receptor binding sites or if the binding of each of these mAbs can cause subtle indirect conformational changes in the RBD should provide insights into the mechanism of synergy.

Finally, by creating closely packed epitope clusters, the virus can elicit non-neutralizing antibodies that will block the binding of neutralizing antibodies. If this mechanism operates *in vivo*, it should provide an explanation for the immune evasion by viruses regardless of whether the virus causes acute or chronic infection. On the other hand, it may also be possible for a host to mount a vigorous neutralizing antibody response against virus infection by producing antibodies that recognize distinct epitopes and act synergistically. Understanding the mechanisms of antibody synergy and interference could serve as a prerequisite to the design of an effective vaccine and the development of effective immune prophylactic treatments against virus infections.

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