

## Human monoclonal antibodies to SARS-coronavirus inhibit infection by different mechanisms

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### ARTICLE INFO

#### Article history:

Received 26 March 2009

Returned to author for revision 22 June 2009

Accepted 15 July 2009

Available online 12 September 2009

#### Keywords:

Severe acute respiratory

syndrome-coronavirus

SARS-CoV

Neutralizing antibodies

S protein

XenoMouse<sup>®</sup>

Human monoclonal antibodies

### ABSTRACT

SARS-CoV causes an acute infection making targeted passive immunotherapy an attractive treatment strategy. We previously generated human mAbs specific to the S1 region of SARS-CoV S protein. These mAbs bind epitopes within the receptor binding domain (RBD) or upstream of the RBD. We show that mAbs recognizing epitopes within the RBD inhibit infection by preventing viral attachment to the cellular receptor. One mAb binds upstream of the RBD and prevents viral entry by inhibiting a post-binding event. Evaluation of several mAbs demonstrated varying ability of the mAbs to select escape mutants when used individually. However, a mixture of antibodies could effectively neutralize a range of mutant viruses. These data strongly suggest that a mixture containing antibodies recognizing distinct regions and targeting more than one step in viral entry is likely to be more effective in neutralizing the virus and suppressing the generation of escape mutants, and thus potentially constitute a highly effective passive immunotherapy.

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### Introduction

Increasingly, RNA viruses appear to be responsible causal agents of newly emerging infections (Duffy et al., 2008; Holmes, 2004; Lai, 2003; Osterhaus, 2008). Severe acute respiratory syndrome-coronavirus (SARS-CoV) is one such emergent infection. Unlike other newly emerged zoonotic infections, SARS-CoV gained the ability to spread efficiently from human to human resulting in a worldwide outbreak (Osterhaus 2008). SARS-CoV causes severe respiratory disease in humans resulting in respiratory distress that requires ventilation support in approximately 20% of infected individuals (Knudsen et al., 2003; Peiris et al., 2004). The global outbreak of SARS-CoV resulted in over 8000 infections worldwide in 2002–2003 (Baker, 2004; Knudsen et al., 2003; Ksizek et al., 2003; Kuiken et al., 2003; Marra et al., 2003). Although, such an outbreak has not recurred, the presence of SARS-CoV in environmental reservoirs remains a threat to human health (Lau et al., 2005; Li et al., 2005; Normile, 2005). Therefore, it is important to establish therapeutic interventions against this potentially life threatening infection.

SARS-CoV causes an acute infection and therefore therapies that offer instantaneous protection are likely to be most effective. A

number of therapeutic strategies such as inhibition of viral entry, viral replication, and viral maturation have been investigated (Golda and Pyrc, 2008; Osterhaus, 2008). Amongst these, inhibition of viral entry is particularly attractive since it will protect target cells from infection thereby reducing the viral load, and allowing the host immune system sufficient time to mount an effective immune response and clear the virus.

The SARS-CoV, mediates viral entry through the S protein. Unlike human CoV-229E, the S protein of SARS-CoV is not cleaved into S1 and S2 domains (Rota et al., 2003). However, it has a distinct S1 domain that mediates receptor binding. The 318–510 amino acid region within S1 represents the receptor binding domain (RBD) that interacts with the cellular receptor, angiotensin-converting enzyme 2 (ACE2) (Li et al., 2005; Li et al., 2003; Wong et al., 2004). The S2 domain mediates fusion between the viral and host membranes (Ksiazek et al., 2003) that is required for releasing the viral genome into the cell (Ingallinella et al., 2004; Li et al., 2005; Li et al., 2003; Rota et al., 2003; Tripet et al., 2004; Wong et al., 2004). Since both binding and fusion events are mediated through the S protein, mAbs that recognize the S protein may inhibit either of these steps.

Previously, we generated a large panel of human monoclonal antibodies using B cells from XenoMouse<sup>®</sup> immunized with SARS-CoV S protein (Coughlin et al., 2007). We identified several epitopes recognized by S1 specific mAbs and found that a majority of these neutralizing antibodies can react with the RBD. The RBD specific antibodies were designated as group 1 based on epitope mapping and further subgrouped based on the antibody complementary deter-

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mining region 3 (CDR3) sequences (Coughlin et al., 2007). We demonstrate that the group 1 monoclonal antibodies (mAbs) neutralize SARS-CoV by preventing association of S protein with the receptor on the surface of the target cells. Regions upstream of the RBD in S1 as well as epitopes within the S2 domain (Coughlin et al., 2007; Duan et al., 2005; Huang et al., 2006; Keng et al., 2005) can also elicit neutralizing antibodies. In this context, we identified one mAb that binds upstream of the SARS-CoV RBD, between amino acids 12 and 261 (Coughlin et al., 2007). We demonstrate that this mAb neutralizes the virus by preventing a post-binding step in viral entry. Furthermore, we show that combinations of antibodies are more effective than individual antibodies in neutralizing the wild type as well as mutant viruses selected in the presence of individual mAbs. These data suggest that targeting more than one epitope and/or step in the viral entry will likely confer better protection against SARS-CoV.

## Results

### *Inhibition of receptor binding by RBD specific mAbs using VeroE6 cells*

We have previously produced human monoclonal antibodies (mAbs) that effectively neutralize SARS-CoV infection by interacting with two distinct regions within the S1 domain of the S protein (Coughlin et al., 2007). In order to understand the underlying mechanism of neutralization by these antibodies, we developed a receptor binding inhibition assay using VeroE6 cells that naturally express the ACE2 receptor on their surface (Li et al., 2003; Wong et al., 2004). The recombinant S protein fragment represented by amino acids 12–510 carrying a human Fc tag (S12–510-Fc) was bound to the surface of target cells at an optimal concentration. The maximal binding of the recombinant protein was detected upon staining with a FITC conjugated anti-human IgG antibody using flow cytometry. The S12–510-Fc recombinant protein was pre-incubated with increasing concentrations of each of the 19 previously identified and characterized S1 specific mAbs (Coughlin et al., 2007) and binding of the protein in the presence of the mAbs was determined and compared to the maximal binding. The IC<sub>50</sub> value for each mAb was determined by the inhibi-

tion curve generated with increasing concentrations of mAb (Table 1). Cells alone, cells lacking the cellular receptor (PA-1), cells mixed with non-ACE2 binding S12–327-Fc fragment, as well as isotype control antibody were included as negative controls to determine non-specific binding and non-specific inhibition. No non-specific binding or non-specific inhibition occurred (Fig. 1A). Representative data obtained for group 1 neutralizing mAbs are shown in Fig. 1A.

Maximum inhibition of binding was seen with the highest concentration of antibodies used (Fig. 1A). Although group 1 mAbs bind within the RBD, based on their complementary determining region 3 (CDR3) sequences they were grouped into various subgroups (e.g. groups 1A1, 1B1, 1B2, 1B3, 1B4, 1D and 1E). These antibodies likely recognize seven distinct epitopes within the RBD and all of them were able to inhibit S12–510-Fc binding (Table 1). The mAbs alone failed to bind significantly to cells (data not shown). Eighteen of the previously identified 19 anti-S1 mAbs efficiently prevented the binding of S1 protein thereby indicating that the mechanism of neutralization of SARS-CoV infection of VeroE6 cells by these antibodies was primarily through the inhibition of virus attachment (Table 1 and Fig. 1A).

### *The mAb that recognizes an epitope upstream of the RBD inhibits a post-binding event*

Limited epitope mapping previously identified a group 2 mAb 4D4 that binds upstream of the RBD (Table 1) (Coughlin et al., 2007). Therefore, we further investigated the mechanism of action of this unique mAb, 4D4. While the RBD specific mAb 1B5 could inhibit receptor association of S12–510-Fc protein, the mAb 4D4 failed to block the interaction of this protein with the cellular receptor (Fig. 1B). In fact, the 4D4 antibody had a marginal enhancing effect on the binding of the S12–510-Fc protein to the cell surface (Fig. 1B).

Since mAb 4D4 could not inhibit binding of the S protein, the ability of mAb 4D4 to disrupt a post-binding step in viral entry was examined using pseudotyped virus that expressed the SARS-CoV S protein on its surface (Huang et al., 2006; Simmons et al., 2004; Yang et al., 2005a; Yi et al., 2005). The post-binding events were detected by binding the pseudovirus to the surface of the target 293T/T17 cells transiently transfected with ACE2 at 4 °C for 1 h, which allowed viral attachment but not entry. Following binding of the pseudovirus, the cells were treated with increasing concentrations of the 4D4 mAb and further incubated at 37 °C overnight. The mAb 4D4 could efficiently prevent viral entry into target cells when added post-pseudovirus binding (Fig. 2). Furthermore, the post-binding inhibition seen was significantly greater than was observed when the mAb and pseudovirus were preincubated prior to addition to the target cells ( $P=0.01$ ).

### *Combinations of mAbs can more efficiently inhibit pseudotyped virus entry*

Next, we investigated whether a mixture of antibodies that react with distinct epitopes and neutralize the virus by different mechanisms can lead to greater inhibition. The mAb 4D4 was used in combination with a representative RBD specific mAb from each group (Coughlin et al., 2007). The use of anti-S1 mAb mixture consisting of two mAbs, resulted in 10–30% greater inhibition of pseudotyped virus entry than each of the mAbs tested alone (Fig. 3). The combinations of mAb showed little to no inhibition of virus in which HIV core was pseudotyped with the VSV G protein (HIV/VSV-G) (data not shown) indicating the specificity of their action.

The 4D4 and 3C7 mAb combination resulted in the most dramatic inhibition as shown by a significant additive effect at the two highest concentrations of mAb used ( $P$  values = 0.0049, 0.046,

**Table 1**

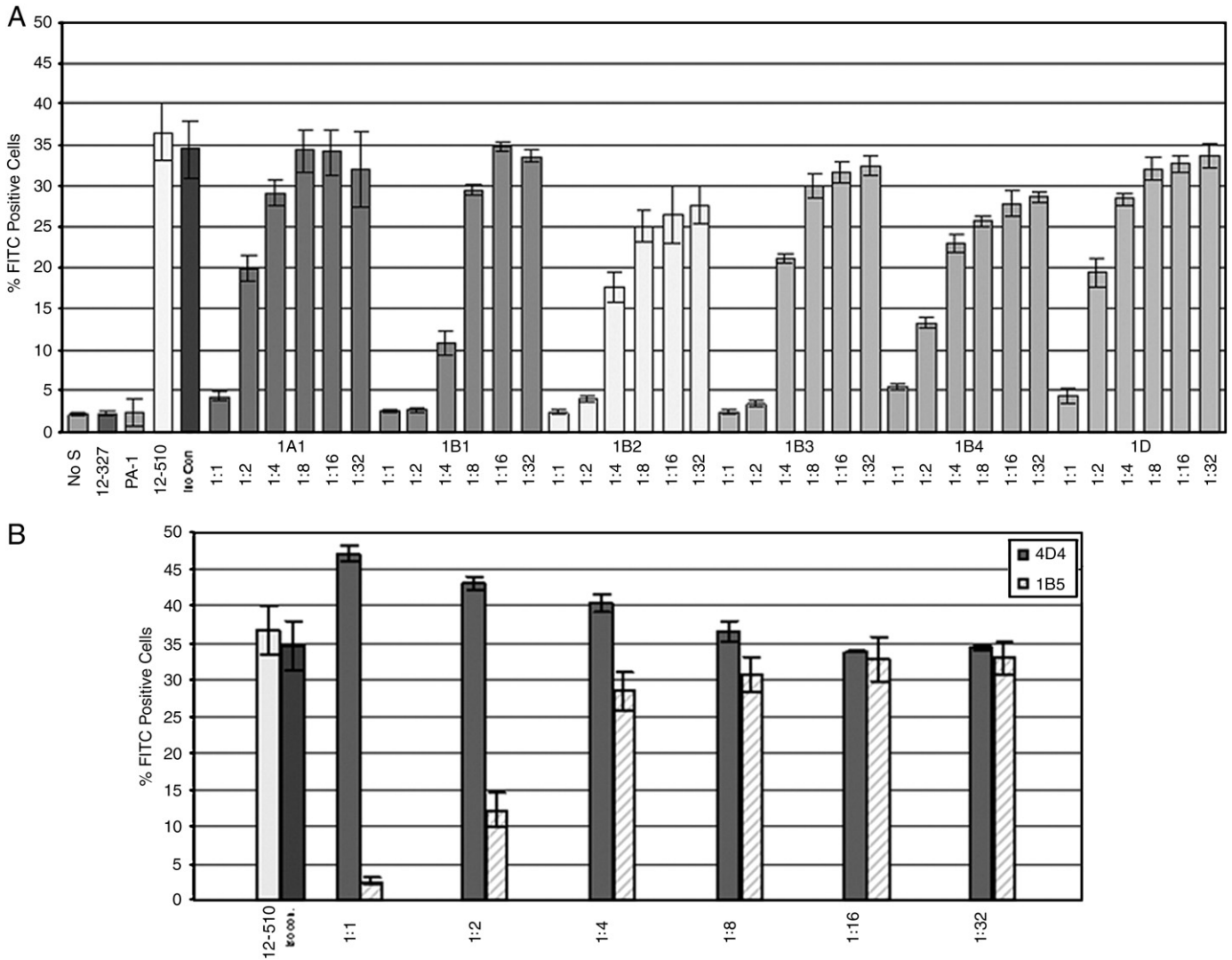
Group <sup>a</sup>	mAb	Binding region	S binding inhibition <sup>b</sup>	IC <sub>50</sub> (µg/ml) <sup>c</sup>
1A1	4E2	RBD	Y	5.31
1A1	4G2	RBD	Y	4.78
1A1	6C1	RBD	Y	4.81
1B1	3A7	RBD	Y	1.17
1B1	5A7	RBD	Y	4.94
1B1	5D3	RBD	Y	1.9
1B1	5D6	RBD	Y	3.17
1B1	6B8	RBD	Y	3.27
1B2	4A10	RBD	Y	6.12
1B2	5A5	RBD	Y	6.79
1B2	3F3	RBD	Y	2.31
1B2	6B5	RBD	Y	2.02
1B2	6C2	RBD	Y	3.53
1B3	5E4	RBD	Y	2.13
1B4	3C7	RBD	Y	4.28
1B4	6B1	RBD	Y	4.08
1D	3H12	RBD	Y	3.71
1E <sup>d</sup>	1B5	RBD	Y	2.84
2B1	4D4	12–261	N	N/A

<sup>a</sup> mAb group determined previously based on ELISA reactivity and immunoglobulin sequence data.

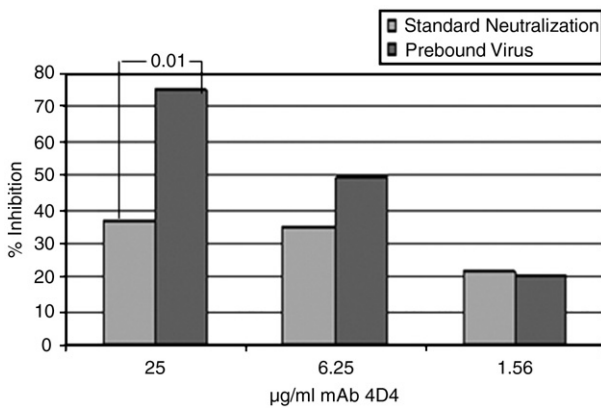
<sup>b</sup> Inhibition of binding of recombinant S protein 12–510-Fc to the surface of target VeroE6 cells.

<sup>c</sup> IC<sub>50</sub> values determined from inhibition curve of the mAb.

<sup>d</sup> mAb 1B5 was previously designated in group 2B2, upon further analysis of the purified mAb 1B5 it was determined to be RBD specific mAb with a unique CDR3 sequence and added to group 1 with designation group 1E.



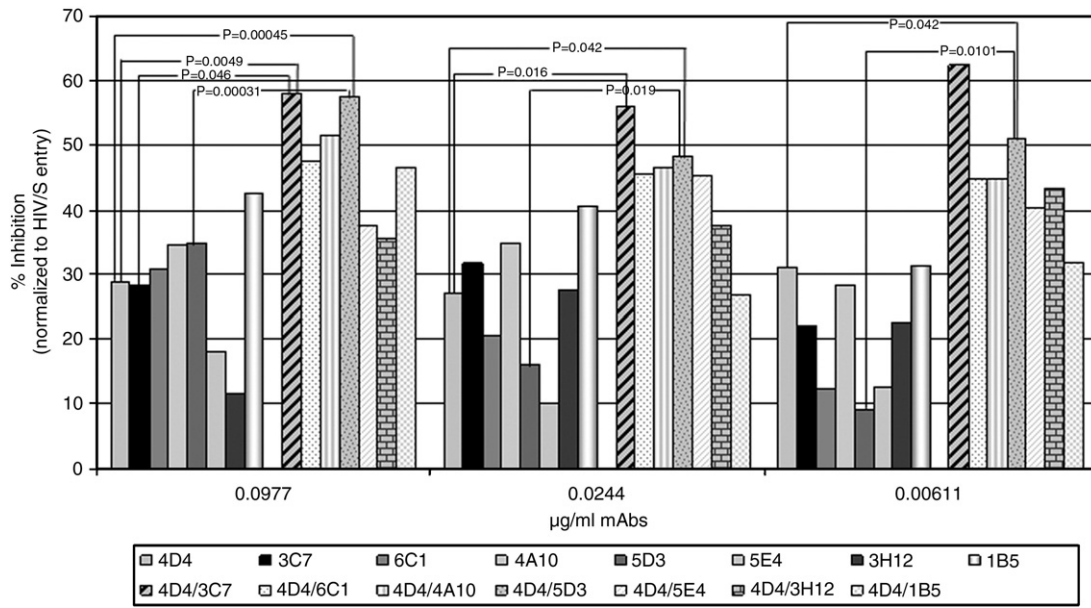
**Fig. 1.** The majority of the human monoclonal antibodies block receptor association. (A) Ability of RBD specific human mAbs (group 1) to inhibit receptor association of S protein was detected using a receptor binding inhibition assay. A representative member is shown for each group of mAbs. The recombinant S12–510-Fc protein was preincubated with increasing dilutions of mAbs. The mixture was added to the target VeroE6 cells and the recombinant protein binding was detected by flow cytometry following staining with an FITC-labeled anti-human IgG. (B) Comparison of the ability of N-terminal specific mAb 4D4 with RBD specific mAb 1B5 to inhibit receptor association of S12–510-Fc protein. Inhibition of receptor association was examined as described for the group 1 mAbs. Isotype: human IgG isotype control.



**Fig. 2.** Post-binding inhibition by mAb 4D4. Increasing concentrations of mAb 4D4 were incubated with HIV/S pseudotyped virus for 1 h at 37 °C and added to target cells in a standard neutralization protocol. Alternatively, HIV/S pseudovirus was bound to target cells at 4 °C for 1 h, and mAb 4D4 was added post-binding at increasing concentrations. Pseudotype virus entry was measured by luciferase expression in target 293T/T17 cells transiently expressing human ACE2. Statistical significance was determined by Student *t*-test.

0.016) (Fig. 3). Another combination that consistently demonstrated increased inhibition was 4D4 and 5D3 (*P* values = 0.00045, 0.00031, 0.042, 0.019, 0.042, 0.0101) (Fig. 3). The combinations of mAbs 4A10, 5E4, or 3H12 with 4D4 demonstrated a significant increase in inhibition (*P* values of 4D4 individually compared to combination, or other mAb used individually compared to combinations equal to 0.0011/0.014, 0.031/0.0404, and 0.0052/0.0062, respectively) when compared to the inhibition seen when each antibody was tested alone at the highest concentrations (Fig. 3). The mAb 1B5 did not show enhanced inhibition when used in combination with 4D4 as compared to the inhibition seen when used individually (Fig. 3).

The cocktail containing 4D4 and 3C7 mAbs was further tested for its ability to neutralize SARS-CoV (Urbani) viral infection of VeroE6 cells. Briefly, 200TCID<sub>50</sub> of SARS-CoV was preincubated with a combination of mAbs 4D4 and 3C7 for 1 h at 37 °C. The virus/antibody mixture was added to VeroE6 cells and cell viability was measured 48 h later. Similar to the results obtained using pseudotyped virus, the combination of 4D4 and 3C7 resulted in significantly increased protection when 0.781 µg/ml or 0.391 µg/ml of antibody was used (Fig. 4).



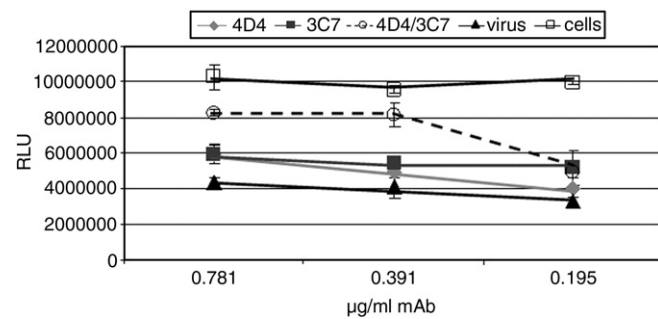
**Fig. 3.** Effects of combinations of anti-S1 RBD specific and 4D4 mAbs on pseudotype virus entry. (A) A representative mAb from each group was mixed with mAb 4D4 at the indicated concentrations and preincubated with pseudotyped virus. Entry was measured by luciferase expression 48 h post-infection in target 293T/T17 cells transiently expressing ACE2. Statistical significance was determined by Student t-test.

*Escape variants are generated with varying frequency in the presence of mAbs, and may be neutralized by other mAbs*

Selection pressures most likely resulted in the genetic changes observed during the outbreak of 2002–2003, which led to the identification of different strains of SARS-CoV (Meulen et al., 2006; Rockx et al., 2007; Sui et al., 2004; Sui et al., 2005; Yang et al., 2005b). Therefore, we tested the ability of eleven anti-S1 mAbs to yield escape mutants. Cultivation of virus in the presence of nine of the eleven mAbs resulted in the emergence of escape mutants at different passages. Passage of virus in the presence of 2EC<sub>50</sub> of three of the mAbs 3C7, 3H12, and 4D4 resulted in the emergence of escape virus within the first several passages (Table 2) perhaps because mutants that could escape neutralization existed within the viral population or mutation occurred early in the virus replication. The existence of escape mutants was confirmed by growing the virus in the presence of higher doses of the corresponding mAb, up to 8EC<sub>50</sub> (Table 2). Other mAbs (5D6, 4A10, 5A5, 5E4, 6B1, and 1B5) resulted in the generation of escape mutants over several passages. Of these mAbs, all except for mAb 4A10 resulted in escape virus observed only in the sub-neutralizing concentration of 1EC<sub>50</sub> (Table 2). Viruses grown in the

presence of either 6C1 or 6C2 did not yield escape mutants at any of the concentrations throughout the nine passages, suggesting the possible recognition of conserved/essential regions within the S1 domain by these antibodies (Table 2).

The ability of other mAbs to inhibit SARS-CoV 3C7 and 4D4 escape variants (SARS-CoV 3C7 and SARS-CoV 4D4, respectively) that were generated in early passages was analyzed using mAbs 3C7, 3H12, and 4D4. SARS-CoV 3C7 and SARS-CoV 4D4 could be effectively neutralized by a mixture of mAbs consisting of 3H12, 4D4 and 3C7 (Figs. 5A and B respectively). Interestingly, mAb 3H12 could moderately neutralize SARS-CoV 3C7 and efficiently neutralize SARS-CoV 4D4 when used individually (data not shown). Similarly, the mAb 3C7 and mAb 4D4 could efficiently neutralize SARS-CoV 4D4 and SARS-CoV

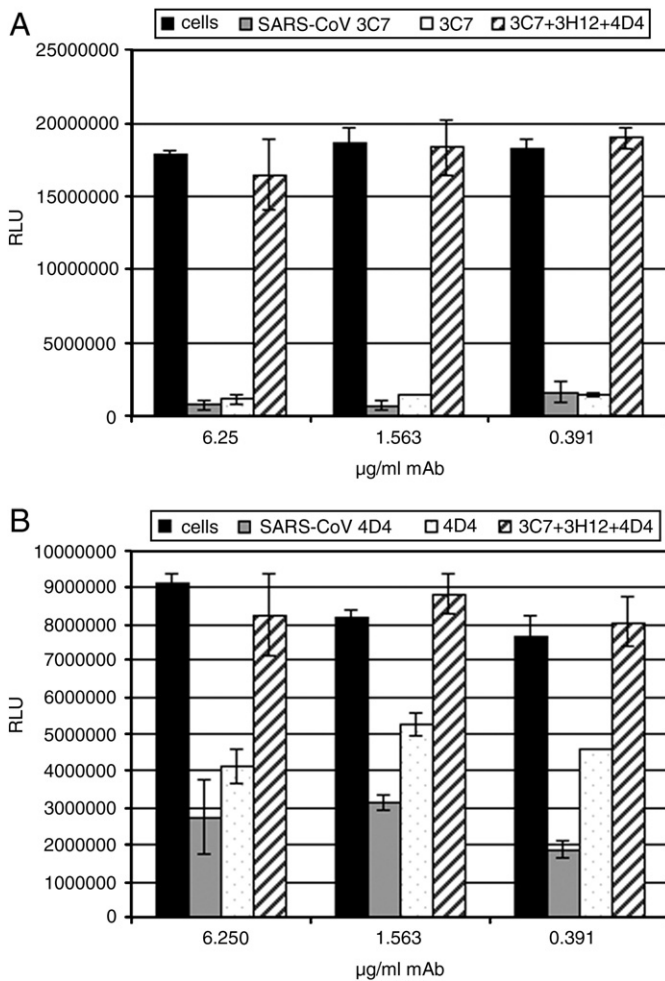


**Fig. 4.** Combination mAbs 4D4 and 3C7 show enhanced inhibition of SARS-CoV (Urbani) infection. The mAbs 4D4 and 3C7 were analyzed for their ability to neutralize SARS-CoV (Urbani) infection in VeroE6 cells. The mAbs (alone or in combination) were preincubated with 200TCID<sub>50</sub> SARS-CoV (Urbani) at 37 °C for 1 h and added to VeroE6 cells. Infection was measured by resulting cell death using the luciferase based Cell TiterGlo assay. Error bars represent standard deviation between duplicates within experiment.

**Table 2**

Passage #		1	2	3	4	5	6	7	8	9
Group	mAb									
1A1	6C1									
1B1	5D6									
1B2	4A10									
1B2	6C2									
1B2	5A5									
1B3	5E4									
1B4	3C7									
1B4	6B1									
1D	3H12									
2B1	4D4									
2B2	1B5									
		Suspected emergence escape mutant 2EC <sub>50</sub>								
		Suspected emergence escape mutant 1.5EC <sub>50</sub>								
		Suspected emergence escape mutant 1EC <sub>50</sub>								
		Escape mutant double mAb concentration								
		Escape mutant confirmed by high mAb concentration (4EC <sub>50</sub> )								
		Escape mutant confirmed by high mAb concentration (8EC <sub>50</sub> )								

SARS-CoV (Urbani) was passaged every 48 h in the presence of mAbs at concentrations of 1, 1.5 and 2 EC<sub>50</sub>. The ability of the mAbs to generate escape mutants was determined by CPE.



**Fig. 5.** Inhibition of SARS-CoV escape mutant virus. SARS-CoV escape mutants were generated by serial passages of the virus in the presence of individual mAbs every 48 h. The neutralizing ability of mAb combination was determined by analysis of cell viability using the luciferase based Cell TiterGlo assay 48 h post-infection. (A) SARS-CoV 3C7 was used in a neutralization assay with 3H12 (group 1) and 4D4 (group 2) mAbs in combination with 3C7. (B) SARS-CoV 4D4 was used in a neutralization assay with mAbs 3H12 and 3C7 (group 1) in combination with mAb 4D4. A single outlier, in both A and B, is not included in the analysis.

3C7, respectively (data not shown). In contrast, the escape variants were not effectively neutralized by the respective mAbs used to generate the variants (Fig. 5). These results showed that despite the propensity of the virus to escape neutralization by some mAbs when used individually, escape mutant viruses were effectively neutralized by a mixture of those antibodies.

## Discussion

In this study, we sought to understand the mechanisms of previously described (Coughlin et al., 2007) anti-S1 mAb mediated neutralization of SARS-CoV, the propensity of a subset of mAbs to generate escape mutants and the efficacy of neutralization of escape variants by a mixture of monoclonal antibodies.

As expected, the mechanism of neutralization by most of these mAbs was the inhibition of receptor binding that prevented viral attachment to the target cells. This observation is consistent with that made by other investigators (Rockx et al., 2008; Sui et al., 2004). The mAb, 4D4 that binds upstream of the RBD, between amino acids 12 and 261 had no significant effect on viral attachment to the target cells but inhibited a post-binding event and prevented viral entry. Recently, a neutralizing antibody, mAb 256, generated from a non-immune scFv library was also found not to inhibit RBD binding (Sui

et al., 2008). However, unlike 4D4 this antibody recognizes an epitope within the RBD. The nAb 256 was demonstrated to have a broader ability to bind to S protein variants, *albeit* with weak neutralizing ability (Sui et al., 2008). The mechanism of inhibition by nAb 256 is not yet known, and the lower efficiency was likely a consequence of selecting the antibody from a library generated using non-immune cells. Therefore, nAb 256 may not have undergone affinity maturation that results from immunization. The mAb 4D4 described here is the first example of an antibody that binds within the N-terminus of the S protein and neutralizes SARS-CoV by inhibiting a post-binding event necessary for viral entry.

Although the function of the N-terminal region of the S1 domain is not clearly defined, a role for this region in mediating dimerization of S protein has been suggested (Tian et al., 2004; Xiao et al., 2004). Additionally, deletion of the first 103 amino acids of the S protein leads to a loss of ability to mediate fusion while retaining ACE2 binding (Tian et al., 2004; Xiao et al., 2003). The ability of mAb 4D4 to more efficiently inhibit viral entry when added post virus binding suggests that the S protein might undergo a conformational change following binding to ACE2 leading to unmasking of the epitope recognized by the 4D4 antibody. These results support the notion that the N-terminus of the S protein contributes to the conformational change required to mediate fusion between the viral and host membranes. The S protein cleavage within the endosome by cathepsin L is essential to initiate fusion of the viral and endosomal membranes (Bosch et al., 2008; Huang et al., 2006; Simmons et al., 2005). Cathepsin L can cleave at amino acid 678 located within the junction of the S1 and S2 domains (Bosch et al., 2008) of the S protein. Other studies have suggested that a conformational change is necessary for the S protein cleavage by cathepsin L (Simmons et al., 2005). Although definitive proof is yet to be generated, it is possible that mAb 4D4 could prevent the conformational change necessary for the S protein cleavage by cathepsin. This speculation is supported by earlier demonstration of the inhibition of the pH-dependent fusion process within the endosome for both Influenza virus and West Nile virus by viral neutralizing antibodies that do not block viral attachment (Imai et al., 1998; Thompson et al., 2009). West Nile virus opsonized by a fusion inhibitory antibody resulted in the retention of the virus in the endosome and likely led to targeted destruction of the virus particle in the lysosomal compartment of the cell (Thompson et al., 2009). These studies suggest that mAbs which can specifically inhibit cathepsin cleavage of specific viral glycoproteins may serve as novel therapies against viruses including Ebola virus, reovirus, and human CoV-229E.

Similar to results obtained using combination mAb therapy against HBV and RSV, a combination of two mAbs targeting different epitopes within the S1 protein of SARS-CoV also has additive effects (Marasco, 2007). Use of mAb 4D4 with another mAb (such as 3C7 or 5D3) resulted in increased inhibition. It is important to note that the combination of mAbs 4D4 and 3C7 conferred near complete protection at an antibody concentration of 0.781 µg/ml, while individual antibodies required about 12.5 µg/ml (Fig. 4). This however is not true for all mAb combinations such as mAbs 4D4 and 1B5. The mAb 1B5 was previously determined to bind upstream of the RBD, however, subsequent analysis determined that the purified mAb 1B5 can in fact bind to the RBD. Lack of synergistic effect indicated that the mAbs 4D4 and 1B5 likely interact with closely positioned conformational epitopes and compete with each other for binding. Similarly, a combination of RBD specific mAbs conferred protection in senescent mice against SARS-CoV challenge; however, the combination was no more efficient than when antibodies were administered individually indicating that the antibodies were possibly competing for binding to the same region (Rockx et al., 2008). These observations highlight the importance of carefully selecting different mAbs to form a therapeutic cocktail.

SARS-CoV is an RNA virus and therefore has a greater intrinsic ability to generate escape mutants, conferred by the error prone RNA

dependent RNA polymerase. Escape mutants were generated rapidly in the presence of high concentrations of three mAbs suggesting that within the viral population antigenic variants existed that escaped mAb neutralization. Although several mAbs did not readily give rise to escape mutants, the escape variants did appear in the presence of suboptimal concentrations of mAbs. This suggested that escape mutants were most likely generated in response to mAb pressure. The escape mutants generated in this study have not yet been sequenced; however, nucleic acid sequence of escape mutants from other studies has revealed that a single point mutation can confer resistance against the selecting antibody (Mitsuki et al., 2008; Sui et al., 2008; ter Meulen et al., 2006). It would be interesting to determine the three dimensional structures of S proteins from mutant viruses and the antibodies used for their selection and relate how the mutation induced change in the conformation of the protein affected its binding to the selecting antibody.

Although virus grown in the presence of monoclonal antibody 3C7, 3H12, or 4D4, could rapidly yield escape mutants; a mixture of these three of mAbs effectively neutralized the escape variants. The mAb 3H12 could neutralize both the SARS-CoV 3C7 and SARS-CoV 4D4 escape variants and confirmed our previous finding that the 3H12 epitope is distinct from epitopes 3C7 and 4D4. Additionally, the 3C7 mAb could neutralize SARS-CoV 4D4, confirming that these two epitopes are also distinct (Coughlin et al., 2007). However, the propensity of these antibodies to allow escape mutants to arise when used alone precludes their individual therapeutic utility. An ideal passive therapy should eliminate or minimize the appearance of escape variants. In this context it is important to note that in the presence of mAbs 6C1 and 6C2 escape mutants failed to arise suggesting that changes in these epitopes might be detrimental to SARS-CoV. Further examination of these epitopes may result in the identification of regions within the S protein that would likely be good candidates for a subunit vaccine.

Although others have demonstrated the utility of mAb cocktails consisting of antibodies with a similar mechanism of action (Meulen et al., 2006; Rockx et al., 2008; Rockx et al., 2007; Sui et al., 2005; Yang et al., 2005b), the present study shows the utility of a cocktail of antibodies not only with different binding specificities but also modes of action. This type of cocktail is likely to neutralize a broader range of variants that may be circulating in nature and suppress the appearance of escape mutants of SARS-CoV when used therapeutically.

## Materials and methods

### SARS-CoV and cells

SARS-coronavirus (SARS-CoV) Urbani strain was obtained from CDC. Virus was propagated in VeroE6 cells in MEM serum free medium (SFM) containing L-glutamine (Gibco, Carlsband, CA). The viral titer was determined by infecting  $1.2 \times 10^4$  VeroE6 cells/well in a 96 well plate with serial 1:10 dilutions of SARS-CoV. After 48 h of incubation at 37 °C in a 5%CO<sub>2</sub> humidified incubator cells were evaluated for cytopathic effect (CPE). The TCID<sub>50</sub> value was calculated by the Reed–Muench method.

Escape virus was generated by serial passage of virus in the presence of various concentrations of individual mAbs (see below). Small aliquots of escape virus was harvested and frozen after each passage at –80 °C. Escape viruses SARS-CoV 3C7 and SARS-CoV 4D4 were propagated in VeroE6 cells in the presence of 2EC<sub>50</sub> of the respective mAbs to maintain the escape mutant. Virus supernatant was harvested after 48 h and frozen in 1 ml aliquots at –80 °C.

### Expression of S proteins

The S1-Fc (Urbani) fragment used to examine mAb activity consisted of amino acids 12–510. The constructs contain a C5 signal

sequence and a human IgG Fc tag, lacking the transmembrane domain (kind gift from Dr. Michael Farzan, Harvard University, (Wong et al., 2004)). The S12-510-Fc construct was transfected into 293T/T17 cells using the CaPO<sub>3</sub> method and purified by protein A sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) as described previously (Coughlin et al., 2007).

### Semiquantitation of inhibition of binding between S1 protein and its receptor

Increasing concentrations of mAb were mixed with a constant concentration (10 µg) of purified recombinant protein S12-510-Fc and preincubated at 4 °C in the presence of 0.5% BSA for 1 h. Following preincubation, the protein/mAb mixture was added to  $2 \times 10^5$  VeroE6 cells and incubated at 4 °C for 1 h. Cells were washed two times with 0.5% BSA. Recombinant protein bound to the cell surface was detected by flow cytometry following staining with an FITC-labeled anti-human IgG (BD Biosciences). Non-specific inhibition was evaluated using an isotype control human IgG2b antibody (Zymed Laboratories, San Francisco, CA).

### Generation of pseudotyped virus

Pseudotyped virus was generated by co-transfection of 293T/T17 producer cells with HIV NL4-3.Luc.R-E- construct and pcDNA3.1-S using Effectene transfection reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Pseudotyped virus allowed for a single round infection. Pseudotyped virus was then used to infect target 293T/T17 cells transiently transfected with ACE2 expression plasmid (human ACE2 expression plasmid was a kind gift from Dr. Michael Farzan).

### Determination of post-binding inhibition

The HIV/S pseudovirus was bound to target 293T/T17 cells transiently transfected with human ACE2 at 4 °C for 1 h, increasing concentrations of mAb 4D4 were added and incubated at 37 °C overnight (approximately 16 h). Subsequently, the medium was replaced and 48 h post-infection luciferase expression was determined. The cells were lysed as per manufacturer's protocol (Promega, Madison, WI) and the lysate mixed with luciferase reagent and read using a luminometer. Simultaneously 4D4 was also used in a standard entry inhibition assay as a positive control for determining the degree of inhibition of pseudotyped virus entry.

### Inhibition of pseudotyped virus entry

Entry inhibition of pseudovirus by standard neutralization was performed by preincubating pseudovirus with mAbs at 37 °C for 1 h. The pseudovirus/mAb mixture or pseudovirus alone was added to target 293T/T17 cells transiently transfected with human ACE2 and incubated overnight at 37 °C (approximately 16 h) after which media was replaced. Pseudotyped virus was quantitated by p24 ELISA (ZeptoMetrix, Buffalo, NY). Following 48 h post-infection cells were lysed and luciferase expression determined as described above.

### Generation of SARS-CoV escape mutants

VeroE6 cells were seeded at  $1 \times 10^4$ /well in a 96 well plate one day prior to infection. The following day, 200TCID<sub>50</sub> of SARS-CoV (Urbani) was preincubated with each of eleven mAbs individually (mAbs 6C1, 5D6, 4A10, 6C2, 5A5, 5E4, 3C7, 6B1, 3H12, 1B5, and 4D4) at concentrations of 1EC<sub>50</sub>, 1.5EC<sub>50</sub>, and 2EC<sub>50</sub> for 1 h at 37 °C. Subsequently, the virus/mAb mixture was added to VeroE6 cells and incubated for 48 h at 37 °C. Following 48 h incubation cells

were examined visually for signs of CPE and virus supernatant passaged in the presence of the mAbs individually at concentrations of 1EC<sub>50</sub>, 1.5EC<sub>50</sub>, and 2EC<sub>50</sub> for 1 h at 37 °C and added to VeroE6 cells plated the day before. Virus supernatant was passaged every 48 h for a total of nine passages.

#### Cell TiterGlo cell viability assay

The Cell TiterGlo Luminescent Cell Viability Assay (Promega, Madison, WI) was used to measure CPE in VeroE6 cells, according to the manufacturer's recommendations. This assay was used to measure the neutralizing capacity of a combination of mAbs 4D4 and 3C7 using SARS-CoV (Urbani). Briefly, 200TCID<sub>50</sub> of SARS-CoV (Urbani) was preincubated with mAbs 4D4 and 3C7 alone and in combination at concentrations of mAb of 0.781, 0.391, and 0.195 µg/ml and a standard microneutralization assay performed using VeroE6 cells plated one day earlier at 1 × 10<sup>4</sup> cells/well (Coughlin et al., 2007). Following 48 h cell viability was measured as luminescence and output expressed as relative luciferase units (RLU) using the Cell TiterGlo Luminescent Cell Viability Assay.

Similarly, the escape viruses SARS-CoV 3C7 and SARS-CoV 4D4 were preincubated with a combination of mAbs (3C7, 3H12, and 4D4) in a standard neutralization assay as described previously (Coughlin et al., 2007). Following 48 h cell viability for the Cell TiterGlo Luminescent Cell Viability Assay was measured as luminescence and output expressed as relative luciferase units (RLU).

#### Acknowledgments

We would like to thank Amgen British Columbia, Inc. for the production of the human antibodies using XenoMouse® and the subsequent purification of these human monoclonal antibodies. We would like to thank Dr. Michael Farzan for providing the recombinant S protein encoding plasmids as well as the human ACE2 encoding plasmid. We would like to thank Dr. Balaji Ganesh for technical support. We would like to thank Dr. Prasad Kanteti for critical reading of this manuscript.

This work was supported by an award from the American Heart Association to M. M. Coughlin.

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