Neutralizing human monoclonal antibodies to severe acute respiratory syndrome coronavirus: target, mechanism of action, and therapeutic potential

Melissa M. Coughlin1 and Bellur S. Prabhakar2*

1Centers for Disease Control and Prevention, Measles, Mumps, Rubella and Herpes Virus Laboratory Branch, Atlanta, GA, USA
2Department of Microbiology and Immunology MC790, College of Medicine, University of Illinois at Chicago, Chicago, IL, USA

SUMMARY

The emergence of Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) led to a rapid response not only to contain the outbreak but also to identify possible therapeutic interventions, including the generation of human monoclonal antibodies (hmAbs). hmAbs may be used therapeutically without the drawbacks of chimeric or animal Abs. Several different methods have been used to generate SARS-CoV specific neutralizing hmAbs including the immunization of transgenic mice, cloning of small chain variable regions from naïve and convalescent patients, and the immortalization of convalescent B cells. Irrespective of the techniques used, the majority of hmAbs specifically reacted with the receptor binding domain (RBD) of the spike (S) protein and likely prevented receptor binding. However, several hmAbs that can bind to epitopes either within the RBD, located N terminal of the RBD or in the S2 domain, and neutralize the virus with or without inhibiting receptor binding have been identified. Therapeutic utility of hmAbs has been further elucidated through the identification of potential combinations of hmAbs that could neutralize viral variants including escape mutants selected using hmAbs. These results suggest that a cocktail of hmAbs that can bind to unique epitopes and have different mechanisms of action might be of clinical utility against SARS-CoV infection, and indicate that a similar approach may be applied to treat other viral infections. Copyright © 2011 John Wiley & Sons, Ltd.

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INTRODUCTION

Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) was identified as a member of the Coronaviridae following an outbreak of acute respiratory syndrome in 2003 [1,2]. Following several super-spreading events, by the end of the outbreak in July of 2003, SARS-CoV infection was responsible for 774 deaths and 8096 cases worldwide involving 29 countries [1]. Virus isolates recovered from individuals in 2003–2004 represented a second zoonotic event indicating a continued threat of SARS-CoV re-entry into humans [3,4]. Molecular analysis of SARS-CoV from the outbreak grouped viruses into early, middle, and late isolates. Early isolates exhibited greater sequence diversity, suggesting that molecular evolution was occurring during the outbreak [4].
Patients infected with SARS-CoV showed atypical pneumonia and severe lung damage. SARS-CoV infected type I and type II pneumocytes, epithelial cells lining the alveolus of the lung [5–11]. Disease progression was accompanied by an influx of inflammatory infiltrates into the lung [12,13]. Approximately 20% of the patients developed acute respiratory distress syndrome (ARDS), and roughly half of the individuals with ARDS died [1,14]. Another unique feature of SARS-CoV relative to other known CoVs was the tissue distribution in infected individuals. SARS-CoV caused systemic infection with the most severe pathology in the lung [1,7,13]. SARS-CoV also replicated in epithelial cells of the intestine and viral RNA was recovered from kidney and liver tissues [13,15].

Genomic analyses and epidemiological data identified palm civets as the intermediate host during the SARS outbreak [16,17]. SARS-like-CoV was isolated by two independent groups from Chinese horseshoe bats [18–20]. Bats serve as the reservoirs of group 1 and group 2 CoVs (SARS-CoV is classified in group 2b) [16,17,21]. Another SARS-CoV outbreak has not been observed, however, the second introduction of SARS-CoV and continued presence of the virus in the animal reservoir indicate that human infection could occur again [3]. Currently, there are no effective targeted treatment options. Viral titers in nasopharyngeal aspirates from infected individuals peaked 10 days post-infection; this provides an opportunity for post-exposure treatment, including passive immunotherapy with anti-SARS-CoV human monoclonal antibodies (hmAbs) [1,22].

THE SPIKE (S) PROTEIN

The spike (S) protein of CoVs mediates binding and fusion events necessary for infection and is the major target of protective immunity [2,4,23,24]. Although the S protein of SARS-CoV shares little amino acid identity (approximately 20%–27%), it shares common structural features with S proteins of other CoVs [2,25]. SARS-CoV S protein is a type 1 transmembrane glycoprotein of approximately 1255 amino acids in length and divided into two functional domains S1 (amino acids 15–680) and S2 (amino acids 681–1255) (Figure 1) [2,25–27]. In many CoVs, the S protein is cleaved during biogenesis and these two functional domains are held together non-covalently; however, like hCoV 229E, the S protein is not cleaved in SARS-CoV [2,25].

The S1 domain forms a globular structure that mediates interaction of the S protein with its receptor, angiotensin-converting enzyme 2 (ACE2) [25,27,28]. A region of S1 consisting of 193 amino acids (amino acids 318–510) is the minimal receptor binding domain (RBD) (Figure 1) [29]. Five cysteine residues within the RBD are important for efficient expression of the RBD and formation of the RBD structure [27]. There are three functional glycosylation sites within the RBD located at amino acids 318, 330, and 357. Expression of S protein requires glycosylation of at least one site; however, glycosylation does not affect ACE2 binding [30].

Co-crystallization of the RBD and human ACE2 identified a concave surface consisting of 70 amino acids (424–494), which contacts the tip of ACE2 and is defined as the receptor binding motif (RBM) (Figure 1) [27]. Basic residues found between amino acids 422 and 463 appeared to be important for mediating the entry of S pseudotyped virus [31]. The alteration of either of the two basic residues, R441A and R453A within this domain, abolished pseudovirus entry [31]. However, the effect of the R441A mutation may be because of decreased protein expression [30]. Interestingly, none of the eight basic residues within the RBM, including R441 and R453, were altered in 96 clinical isolates (Figure 2) [31]. Two residues, R426 and N473, were identified as critical for ACE2 binding [30]. Changes in the RBM, namely N479K and T487S (Figure 2), might have allowed more efficient binding to human ACE2 [3,17,27,32].

The S2 domain mediates fusion and contains the putative fusion peptide and two conserved helical regions (HR1 and HR2) that upon cleavage by the endosomal protease cathepsin L form the six helix bundle fusion core (Figure 1) [2,25,33–37]. These two regions are connected by a long (170 amino acid) inter-domain loop likely allowing flexibility to facilitate fusion [25]. The HR1 domain forms the inner helical coiled coil region onto which the HR2 domain associates in an anti-parallel manner to form the fusion core [25,34,35,37]. This brings the putative fusion peptide (770–788) in close proximity to the transmembrane domain and facilitates juxtaposition of the cellular and viral membranes required for fusion [37,38]. Therapies that disrupt interactions of the HR1 and HR2 domains would likely confer protection, and synthetic HR2 peptides have been demonstrated to block SARS-CoV infection, however, HR1 peptides have not been
Figure 1. Severe acute respiratory syndrome coronavirus (SARS-CoV) spike protein and monoclonal antibody (mAb) epitopes. Depiction of various functional domains of SARS-CoV S protein. Receptor binding domain (RBD), the minimum region responsible for binding angiotensin-converting enzyme 2 (ACE2) [29]; receptor binding motif (RBM), specific region contacting ACE2 [27]; fusion peptide (FP), HR1, and HR2 helical repeat domains involved in fusion [27,37,38]. mAbs identified to react with indicated regions. *RBD specificity based on mutations identified in escape variants. **Influence of Y777D mutation identified in escape variants

Figure 2. Alignment of representative severe acute respiratory syndrome coronavirus (SARS-CoV) clinical isolates. The S protein sequences of 94 SARS-CoV clinical isolates were aligned using Jellyfish software. The changes within the S1 domain are depicted by representative isolates. The magnification highlights changes within the receptor binding domain. The box highlights amino acids that fall within the defined receptor binding motif
found to inhibit entry [37]. Abs targeted to S2 can disrupt viral entry (discussed below).

The cellular glycoprotein CD209L (L-SIGN), also found on type II pneumocytes, has been described as an alternate receptor or a co-receptor for SARS-CoV [39]. Infectivity mediated by L-SIGN, albeit at a significantly lower efficiency than ACE2, has been demonstrated in non-permissive cells [39,40]. Residues important for S interaction with L-SIGN vary from the defined RBD, with several residues mapping N-terminal to the RBD. Neutralizing Abs that interrupt the association of S and ACE2 fail to prevent L-SIGN mediated entry [40]. The interaction of S protein with dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) can facilitate the entry of SARS-CoV via ACE2. This interaction domain of S protein is between amino acids 324 and 386 within the minimum RBD, but N-terminal to the RBM [41]. DC-SIGN has also been implicated in the ability of dendritic cells (DCs) to transfer SARS-CoV to target cells in vitro without productively infecting the DCs, similar to HIV [42].

SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS AND HUMORAL IMMUNITY

The humoral immune response appeared to play the primary role in recovery from SARS-CoV infection. SARS-CoV induced neutralizing Abs, primarily targeted to the RBD, which positively correlated with disease outcome in infected individuals [43–53]. A DNA vaccine study in BALB/c mice showed that the antibody (Ab) response was responsible for reduced viral replication and passive transfer of immune serum to naïve mice conferred protection against SARS-CoV challenge. Depletion of CD4+ and CD8+ T cells did not affect vaccine protection and adoptive transfer of CD4+ and CD8+ T cells from immunized mice failed to protect naïve mice against SARS-CoV challenge. These studies demonstrated the importance of Abs in the clearance of SARS-CoV infection and the potential utility of passive immunotherapy against SARS-CoV [54].

ANTIBODY NEUTRALIZATION

There are three main ways that Abs elicit their protective activities, neutralization, opsonization, and complement activation [55]. There are several mechanisms described for the neutralization of viruses by Abs. Abs may (i) aggregate viruses preventing binding and entry; (ii) bind to the viral attachment protein or the cellular receptor and prevent entry; (iii) prevent conformational changes necessary for fusion; (iv) destabilize the virus and cause the release of viral nucleic acid outside the cell; (v) prevent uncoating of the virus capsid; or (vi) prevent the release of progeny virus from infected cells [56–58].

The constant (C) region of the Ab can contribute to viral clearance through opsonization or complement activation [59]. However, Ab-dependent enhancement (ADE) of infection may occur through the uptake of viral particles via complement receptors or Fc receptors (FcR). ADE has been demonstrated for the group 2 CoV feline infectious peritonitis virus (FIPV) [60,61]. There is some evidence to suggest that ADE may occur with SARS-CoV (discussed below).

MONOCLONAL ANTIBODY TECHNOLOGY

The pharmaceutical development of mAbs has expanded greatly, and mAbs can provide a powerful mode of therapeutic intervention with a highly specific treatment [55,57,62]. This is particularly important for viruses in which the neutralizing Ab response is important for protection, as described for SARS-CoV [56,62].

A mouse mAb targeting CD3 on human T cell was first approved for therapeutic use to prevent kidney transplant rejection [63]. However, treated individuals developed an immune response against the mouse C region limiting its use. To circumvent this, therapeutic chimeric and hmAbs have been produced (Table 1) [63]. Currently, hmAbs can be generated using several strategies, including preparation of hybridomas (using a transgenic mouse), phage display technologies, and the immortalization of convalescent B cells (Table 1) [63–65]. All three strategies have been used to produce hmAbs against SARS-CoV [66–69].

The use of a human immunoglobulin transgenic mouse (e.g., XenoMouse®) allows production of high affinity hmAbs through hybridoma fusion. In the immunized mouse, immunoglobulin genes undergo affinity maturation because of somatic hypermutation [63,70,71]. Additionally, transgenic mice are available in the five immunoglobulin classes, allowing selection of the most appropriate C region required to mediate a particular function [63].

Human mAbs may also be generated from immunoglobulin cDNA libraries corresponding to the immunoglobulin variable (V) regions. However,
<table>
<thead>
<tr>
<th>Name1</th>
<th>Components</th>
<th>Approval date2</th>
<th>Treatment</th>
<th>Comments</th>
</tr>
</thead>
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<td>Orthoclone OKT3</td>
<td>Mouse</td>
<td>9/1992</td>
<td>Target human CD3 prevention kidney rejection and steroid resistant cardiac and hepatic rejection</td>
<td>First mouse mAb approved, patient immune response decreased efficacy</td>
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<td>Rituxan (rituximab)</td>
<td>Chimeric</td>
<td>11/1997</td>
<td>Arthritis and lymphoma (CD20+ B cells)</td>
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<tr>
<td>Zenapax (daclizumab)</td>
<td>Humanized</td>
<td>12/1997</td>
<td>Prophylaxis of acute kidney transplant rejection (anti-IL-2R)</td>
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<tr>
<td>Simulect (basiliximab)</td>
<td>Chimeric</td>
<td>5/1998</td>
<td>Prophylaxis of acute kidney transplant rejection (anti-IL-2R)</td>
<td></td>
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<tr>
<td>Synagis (palivizumab)</td>
<td>Humanized</td>
<td>6/1998</td>
<td>RSV in infants</td>
<td>Only approved mAb for viral disease</td>
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<tr>
<td>Remicade (infliximab)</td>
<td>Chimeric</td>
<td>8/1998</td>
<td>Crohn’s disease and arthritis (anti-TNF-α)</td>
<td></td>
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<td>Campath (alemtuzumab)</td>
<td>Humanized</td>
<td>5/2001</td>
<td>B-cell chronic lymphocytic leukemia (B-CLL) (anti-CD52)</td>
<td>Cytolytic</td>
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<td>Zevalin (ibritumomab-tioxetan)</td>
<td>Mouse</td>
<td>2/2002</td>
<td>Relapsed or refractory non-Hodgkin’s lymphoma (anti-CD20)</td>
<td>Targeted radioimmunotherapy</td>
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<tr>
<td>Humira (adalimumab)</td>
<td>Human (phage display)</td>
<td>12/2002</td>
<td>Crohn’s disease, rheumatoid arthritis, ankylosing spondylitis (anti-TNF-α)</td>
<td>First human monoclonal antibody approved [64]</td>
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<tr>
<td>Bexxar (tositumomab)</td>
<td>Mouse</td>
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<td>CD20 expressing relapsed or refractory non-Hodgkin’s lymphoma (anti-CD20)</td>
<td>Targeted radioimmunotherapy</td>
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<td>Xolair (omalizumab)</td>
<td>Humanized</td>
<td>6/2003</td>
<td>Moderate to severe persistent asthma (anti-IgE)</td>
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<td>Avastin (bevacizumab)</td>
<td>Humanized</td>
<td>2/2004</td>
<td>Metastatic colorectal cancer (anti-VEGF)</td>
<td>Anti-angiogenesis</td>
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<td>Erbitux (cetuximab)</td>
<td>Chimeric</td>
<td>2/2004</td>
<td>Metastatic colorectal cancer (anti-EGFR)</td>
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<td>Type</td>
<td>Approval Date</td>
<td>Indication</td>
<td>Description</td>
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<td>Mylotarg</td>
<td>Humanized</td>
<td>1/2006</td>
<td>CD33⁺ acute myeloid leukemia (AML) in 1st relapse or non-chemotherapeutic candidate (anti-CD33)</td>
<td>Conjugated anti-tumor antibiotic</td>
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<td>Lucentis</td>
<td>Humanized</td>
<td>6/2006</td>
<td>Neovascular (wet) age-related macular degeneration and macular edema following retinal vein occlusion (anti-VEGF-A)</td>
<td>Conjugated anti-tumor antibiotic</td>
</tr>
<tr>
<td>Vectibix</td>
<td>Human (transgenic mouse)</td>
<td>9/2006</td>
<td>EGFR-expressing colorectal cancer</td>
<td>First human monoclonal Ab produced in transgenic mice to receive approval</td>
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<td>Soliris</td>
<td>Humanized</td>
<td>3/2007</td>
<td>Paroxysmal nocturnal hemoglobinuria (PNH) (anti-complement C5)</td>
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<td>Simponi</td>
<td>Human (transgenic mice)</td>
<td>4/2009</td>
<td>Rheumatoid arthritis, active psoriatic arthritis, active ankylosing spondylitis (anti-TNF-α)</td>
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<td>Ilaris</td>
<td>Human (transgenic mouse)</td>
<td>6/2009</td>
<td>Cryopyrin-associated periodic syndromes (CAPS) (anti-IL-1β)</td>
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<tr>
<td>Stelara</td>
<td>Human (transgenic mouse)</td>
<td>9/2009</td>
<td>Moderate to severe plaque psoriasis (anti-IL-12, 23)</td>
<td>Targets p40 subunit</td>
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<td>Arzerra</td>
<td>Human (transgenic mouse)</td>
<td>10/2009</td>
<td>Chronic leukocytic leukemia (anti-CD20 cytolytic)</td>
<td>For treatment of CLL refractory to fludarabine and alemtuzumab</td>
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<tr>
<td>Actemra</td>
<td>Humanized</td>
<td>1/2010</td>
<td>Moderate to severe rheumatoid arthritis (anti-IL-6)</td>
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<td>Prolia and Xgeva</td>
<td>Human (transgenic mouse)</td>
<td>6/2010</td>
<td>Prolia: osteoporosis in postmenopausal women at high risk Xgeva: prevention of skeletal-related events in solid tumor bone metastases (anti-RANKL)</td>
<td>Xgeva approved 11/2010</td>
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<tr>
<td>Benlysta</td>
<td>Human (phage display)</td>
<td>3/2011</td>
<td>Autoantibody positive systemic lupus erythematosus (anti-BLyS)</td>
<td></td>
</tr>
</tbody>
</table>

Abs, antibodies; mAbs, monoclonal antibodies; RSV, respiratory syncytial virus; VEGF, vascular endothelial growth factor; EGFR, epidermal growth factor receptor; RANKL, receptor activator of NF-kappaB ligand.

1This table is comprised only of complete mAbs and does not include Fab fragment monoclonal therapies or discontinued or diagnostic mAbs.

2Monoclonal Abs currently approved for treatment as of March 2011 [64], [65].
recombinant hmAbs generated from a naïve cDNA library, will not have undergone affinity maturation, and can be of low affinity [63]. Monoclonal Abs produced in bacteria are unlikely to be fully glycosylated, and are usually contaminated with lipopolysaccharide (LPS) and not desirable for human use [62]. Therefore, recombinant hmAbs require expression in mammalian cells.

Some of the above mentioned limitations can be overcome by the use of memory B cells obtained from convalescent patients, which have likely undergone affinity maturation. Immortalization of such memory B cells with EBV has been used to generate hmAbs against SARS-CoV and avian influenza [57,69]. Cells transformed with EBV in the presence of a polyclonal activator such as CpG oligonucleotide significantly increased the efficiency of immortalization of the memory B cells [69].

Immunoglobulin gene sequence analysis of SARS-CoV S specific hmAbs generated using XenoMouse® (Amgen Fremont Inc, Freemont, CA, USA) indicated a preferential usage of certain heavy (H) chain genes, from the VH1 and VH3 gene families, and light (L) chain genes [66]. SARS-CoV specific non-immune single-chain variable antibody fragments (scFv) isolated by phage display also showed preferential usage of VH1 and VH3 gene families [68].

HUMAN MONOCLONAL ANTIBODIES AGAINST SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS

Most neutralizing hmAbs against SARS-CoV bound to the RBD [23,43,46,47,66–69,72,73]. The RBD was also the dominant domain recognized by Abs in infected patients [23]. Neutralizing hmAbs targeting N-terminal (amino acids 12–261, 130–150) and C-terminal of the RBD (amino acids 548–567, 607–627), close to the junction of the S1 and S2 domains (amino acids 789–799, 803–828) and within the HR2 domain, have been reported [49,66,67,74–76]. Additionally, a mouse mAb that interrupted the capture of S pseudotyped virus by DC-SIGN and reduced trans-infection has also been reported [41].

Two strains of transgenic mice have been used to produce hmAbs against SARS-CoV [66,67]. The difference between these strains of mice is that the mouse L chain genes are still functional in the Medarex HuMAb-Mouse® (Bristol-Myers Squibb, New York City, NY, USA), and therefore these mice can produce chimeric mAbs. The XenoMouse® from Amgen has all the mouse L chain genes deleted, and B cells produce only human Abs. Immunization of the XenoMouse® with recombinant S protein ectodomain resulted in identification of 27 SARS-CoV neutralizing hmAbs [66]. These S1 specific hmAbs fell into one of the eight groups determined by differences in binding to S protein fragments and immunoglobulin gene sequences [66]. The majority of the S1 specific neutralizing Abs reacted with the RBD; however, one mAb, 4D4, reacted N-terminal of the RBD between amino acids 12 and 261 (Figure 1 and Table 2) [66]. Immunization of HuMAb® mice resulted in the production of 36 hybridomas from which two neutralizing mAbs were further evaluated. Monoclonal Ab 201 mapped to the RBD, recognizing an epitope containing amino acids 490–510 (Figure 1 and Table 2). Monoclonal Ab 68, a chimeric mAb expressing a mouse light chain, mapped to amino acids 130–150 N-terminal of the RBD (Figure 1 and Table 2) [67]. Monoclonal Ab 201, when given prophylactically, reduced viral titers in the lungs of mice below the limit of detection and reduced viral titers and pathology in the lungs of Golden Syrian hamsters when given 24 h post infection (Table 2) [67,77].

Screening of immune and non-immune scFv libraries was also used to generate SARS-CoV specific hmAbs. Screening of non-immune scFv libraries using whole virus and recombinant S1 proteins led to the identification of SARS-CoV specific Abs [68,78]. One of the eight S1 domain specific Abs, mAb 80R neutralized SARS-CoV and demonstrated 20 times the neutralizing capacity of the monovalent scFv (Table 2) [68]. The mAb 80R recognized a glycosylation independent epitope within amino acids 426–492 of the S protein (Figure 1 and Table 2) [68,79]. Three of the four S specific neutralizing scFvs identified by van den Brink et al. recognized the same or overlapping conformational epitope (s) within the RBD, with mAb CR3014 demonstrating the strongest affinity (Figure 1). MAb CR3014 bound 7 of 8 recombinant RBD proteins representing isolate variants, and was also able to reduce viral titers in the lungs of infected ferrets, and prevent viral shedding in the nasopharynx and pathological lesions in the lungs (Table 2) [78,80].

The immortalization of B cells from convalescent patients yielded several neutralizing hmAbs that reacted with the RBD. The mAbs could be
divided into six groups based on differential neutralization of SARS-CoV variants. The hmAb S3.1 decreased viral titer in the lungs of infected mice, and group VI mAbs demonstrated protection against lethal challenge in a mouse model (Table 2) [69,81].

**Mechanism of action of SARS-CoV neutralizing hmAbs**

Most RBD specific hmAbs neutralized by blocking S protein binding to ACE2 [67,68,74,78]. However, some neutralizing Abs that bound within the RBD appeared not to use the same mechanism of inhibition as other RBD specific hmAbs. For example, hmAbs 256 and CR3022, identified independently from scFv libraries, did not inhibit receptor binding (Table 2) [79,82]. Interestingly, hmAb 256 enhanced binding of the S protein of the GD03 strain, a 2003/2004 isolate, to the surface of target cells but was still neutralizing [79]. The identification of neutralizing hmAbs that bind N-terminal of the RBD (mAb 4D4 and mAb 68) has led to interesting insights into the function of this region and the identification of epitopes distant from the RBD [66,67,79]. The presence of these neutralizing epitopes demonstrated an important, yet undefined role of this region in SARS-CoV entry. The hmAbs 4D4 and 68 do not prevent association of the S protein with the cellular receptor (Table 2) [67,74]. Deletions within the first 300 amino acids of S1 eliminated fusion ability of S protein in a cell-to-cell fusion assay while maintaining receptor binding function [83]. The N-terminal region of S protein also seems to play a role in the trimerization of the S protein, a structure that may be required for fusion [83]. Therefore, binding of hmAbs 4D4 or 68 to this region may interfere with virus fusion. Another potential role for this region is that it may interact with an unidentified co-receptor required for entry, or it may disrupt the interaction of S protein with L-SIGN [40]. Comparison of mAb 4D4 with 12–261 specific non-neutralizing Abs identified in the same screening could further elucidate the role of this region in viral entry [66].

Further study of hmAb 4D4 revealed that it prevented a post-binding step in viral entry, and the addition of hmAb 4D4 post virus binding to cells efficiently neutralized S pseudotyped virus entry [74]. This is an important discovery, because the inclusion of these hmAbs in a therapeutic cocktail allows targeting of more than one entry step. Non-S1 reactive hmAbs also play an important role in the inhibition of viral entry. Screening of full-length S reactive, but S1 non-reactive, hmAbs produced in the XenoMouse® resulted in the identification of 56 neutralizing Abs. These Abs may recognize an epitope within the S2 domain, and may neutralize by inhibiting fusion [74]. The S2 domain is more conserved than the S1 domain, and therefore, S2 region reactive hmAbs when combined with distinct S1 reactive hmAbs may be more effective against a wide range of clinical isolates [2,49,74,76]. Furthermore, it would be interesting to test the S2 specific hmAbs against zoonotic SARS-CoV isolates and bat SARS-like-CoV isolates to determine possible cross-reactivity. There appears to be little cross-reactivity between human SARS-CoV and bat SARS-like-CoV isolates, likely because of large differences in the RBD, however, neutralization of human/bat S chimera pseudotyped virus demonstrated some cross-neutralization potential outside the RBD [84].

**Neutralization of severe acute respiratory syndrome coronavirus isolates and escape mutants; potential use of combination human monoclonal antibody therapy**

Immunoglobulin gene sequencing of XenoMouse® hmAbs identified eight unique Ab groups recognizing conformational epitopes within the RBD [66]. Similarly, the panel of hmAbs generated from the immortalization of convalescent patient B cells may be divided into six groups based on their ability to neutralize isolates from early, middle, and late phase of the 2002/2003 SARS outbreak and zoonotic isolates [81]. Minor alterations in the RBD structure could result in the loss of conformational epitopes, allowing neutralization escape by cognate hmAbs. Examination of the sequences of clinical isolates demonstrated that changes within the RBD of S protein are tolerated (Figure 2). Interestingly, the majority of changes in RBD (through alanine scanning) did not result in significant loss of receptor binding, demonstrating the flexibility of S protein to escape immunological pressure without losing infectivity [30]. Furthermore, single amino acid changes within the S protein can abolish neutralization by a given mAb [78,81]. Monoclonal Abs generated against late isolates may not be effective against early zoonotic isolates and may lead to enhanced infection. This was demonstrated by the increased entry mediated by pseudovirus
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Monoclonal technology</th>
<th>Antibody class</th>
<th>Binding region</th>
<th>Efficacy in vitro</th>
<th>Animal models</th>
<th>Efficacy in vivo</th>
<th>Mechanism of action</th>
<th>References</th>
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<tr>
<td>Group 1A1 (4E2, 4G2, 6C1)</td>
<td>XenoMouse®</td>
<td>IgG2</td>
<td>318–510</td>
<td>0.781 g/ml</td>
<td>NT</td>
<td>ND</td>
<td>Inhibition receptor binding</td>
<td>63,71</td>
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<td>IgG2</td>
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<td>0.195–0.781 g/ml</td>
<td>NT</td>
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<td>Inhibition receptor binding</td>
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<td>12.5 g/ml</td>
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<td>3.125–12.5 g/ml</td>
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<td>ND</td>
<td>Inhibition receptor binding</td>
<td>63,71</td>
</tr>
<tr>
<td>Group 1E (1B5)</td>
<td>XenoMouse®</td>
<td>IgG2</td>
<td>318–510</td>
<td>0.195 g/ml</td>
<td>NT</td>
<td>ND</td>
<td>Inhibition receptor binding</td>
<td>63,71</td>
</tr>
<tr>
<td>Group 2B1 (4D4)</td>
<td>XenoMouse®</td>
<td>IgG2</td>
<td>12–261</td>
<td>12.5 g/ml</td>
<td>NT</td>
<td>ND</td>
<td>Inhibition post binding step</td>
<td>63,71</td>
</tr>
<tr>
<td>201</td>
<td>HuMAb-Mouse®</td>
<td>IgG1</td>
<td>490–510</td>
<td>37.5nM</td>
<td>Mouse Golden Syrian Hamster</td>
<td>Reduced viral titers in lung 2d p.i. (1.6–40 mg/kg) Reduced pathology in hamster model</td>
<td>Inhibition receptor binding</td>
<td>64,74</td>
</tr>
<tr>
<td>68</td>
<td>HuMAb-Mouse®</td>
<td>IgG1 mouse</td>
<td>130–150</td>
<td>Not 100% protection</td>
<td>Mouse</td>
<td>Reduced viral titers</td>
<td>Does not inhibit</td>
<td>64,74</td>
</tr>
<tr>
<td>Group</td>
<td>Description</td>
<td>IgG Concentration</td>
<td>receptors binding</td>
<td>Mouse VIRAL TITERS</td>
<td>receptor binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>--------------------------------------------------</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>80R</td>
<td>Human non-immune scFv</td>
<td>0.37 nM</td>
<td>50%</td>
<td>Reduced viral titers in lung 2d p.i. (1.6–40 mg/kg)</td>
<td>Inhibition receptor binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR0314</td>
<td>Human non-immune scFv</td>
<td>5.2 g/ml</td>
<td>50%</td>
<td>Reduced viral titers in lung 2d p.i. (20–500 g/ml)</td>
<td>Inhibition receptor binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CR0322</td>
<td>Human non-immune scFv</td>
<td>23.5 g/ml</td>
<td>50%</td>
<td>Reduced viral titers in lung and eliminated lung pathology (10 mg/kg)</td>
<td>ND Does not inhibit receptor binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group I (S132, S228.11)</td>
<td>EBV transformed B cells</td>
<td>196–1984 ng/ml 100PFU</td>
<td>50%</td>
<td>Reduced viral titers in lung 2d p.i. (100 pfu)</td>
<td>Inhibition receptor binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II (S117.7, S224.17)</td>
<td>EBV transformed B cells</td>
<td>154–194 ng/ml 100PFU</td>
<td>50%</td>
<td>Reduced viral titers in lung 2d p.i. (100 pfu)</td>
<td>Inhibition receptor binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group III (S3.1, S127.6, S217.4, S222.1, S237.1)</td>
<td>EBV transformed B cells</td>
<td>8–65 ng/ml 100PFU</td>
<td>50%</td>
<td>Reduced viral titers in lung 2d p.i. (100 pfu)</td>
<td>Inhibition receptor binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group IV (S110.4, S218.9, S223.4, S225.12, S226.10, S231.19, S232.17, S234.6)</td>
<td>EBV transformed B cells</td>
<td>9–90 ng/ml NT 100PFU</td>
<td>50%</td>
<td>Reduced viral titers in lung 2d p.i. (100 pfu)</td>
<td>Inhibition receptor binding</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group V (S124.5, S219.2)</td>
<td>EBV transformed</td>
<td>ND</td>
<td>50%</td>
<td>Reduced viral titers in lung 2d p.i. (100 pfu)</td>
<td>Inefficient inhibition</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Continues
bearing the S protein of the palm civet isolate by hmAbs S3.1, S127, and S111 [85]. Therefore, neutralizing hmAbs that can bind to conserved epitopes expressed on a wide range of isolates are the best candidates for passive immunotherapy.

Escape mutants arise when the virus is grown in the presence of hmAbs [74,81,82,86]. Several escape mutants of SARS-CoV were selected in early passages in the presence of neutralizing quantities of XenoMouse® hmAbs (3C7, 3H12, and 4D4). Escape mutants also appeared in later passages in the presence of sub-neutralizing amounts of hmAbs. No escape mutants were found when the virus was grown to nine passages in the presence of hmAbs 6C1 and 6C2 [74]. Escape mutants, with single amino acid changes, arose in the presence of hmAbs S109.8 and S230.15, but hmAb S227 produced escape mutants only against a zoonotic isolate [81,86]. Virus grown in the presence of the hmAb developed from a non-immune scFv library CR3014 also yielded escape mutants with a single amino acid change P462L [82]. Virus grown in the presence of a hmAb generated from an immune scFv library CR3022 did not yield escape mutants [82]. Inability to generate escape mutants in the presence of certain hmAbs would suggest that these regions are critical for virus infectivity. However, as demonstrated for the hmAbs 6C1, 6C2, S228.11, S111.7, and S224.1, variants can also escape neutralization by hmAbs other than those used for their selection [74,81,86]. However, escape variants generated in the presence of one Ab could be neutralized by a different hmAb or a combination of hmAbs. These findings highlight the importance of using a mixture of hmAbs that may neutralize a wide range of variants through different mechanisms by targeting different epitopes for passive immunotherapy [74,81,82,86].

The hmAb 4D4 has been identified to inhibit a post-binding step in viral entry and bind N-terminal of the RBD. This mAb was studied in combination with RBD specific hmAbs using pseudotyped virus. When used in combination with other hmAbs, such as 3C7, there was an increased efficacy of neutralization over the individual hmAbs. This increased efficacy was confirmed in a neutralization assay with SARS-CoV [74]. Similarly, the hmAbs CR3014 and CR3022 demonstrated synergy when used in combination to neutralize SARS-CoV. Interestingly, the combination of CR3014 and CR3022 was more effective against CR3014 escape virus than CR3022.
alone [82]. However, certain combinations, such as hmAbs 4D4 and 1B5, did not yield increased efficacy [74]. Ideally, the design of a therapeutic cocktail should prevent any competition between hmAbs and allow for the hmAbs to work together to increase inhibition beyond that seen with individual hmAbs.

Additionally, the examination of the activity of mAb combinations may identify possible candidates that may be engineered to generate higher affinity Abs [63,79]. The mAb 80R was modified to broaden the neutralizing capability using two methods, light chain shuffling, and focused mutagenesis. The D480A/G mutation within S protein was found to render mAb 80R ineffective. The crystal structure of 80R when bound to SARS-CoV RBD revealed a key residue (R162) in the CDR1 region of the 80R κ light chain that likely plays a role in the loss of neutralization when amino acid 480 is mutated. This region was identified as a “hot spot” for activation-induced cytidine deaminase and was targeted to generate hmAbs of broader reactivity. Both methods identified several hmAbs capable of broader neutralization demonstrated by their ability to neutralize a late 2002/2003 isolate, a 2003/2004 isolate, and isolates with D480A/G change [79]. The examination of the differences in relative binding affinities and neutralizing efficiencies of hmAb 80R allowed the engineering of a higher affinity hmAb; an approach that may be applied to other promising hmAbs.

When targeting an RNA virus like SARS-CoV, it is important to consider the mutability of the virus target caused primarily by the error prone RNA-dependent RNA polymerases. Despite the unusually large RNA genome of CoVs (27kb to nearly 32kb), they have an error frequency similar to that noted in other RNA viruses and can be predicted to incorporate three changes per genome per replication cycle [17,87]. Interestingly, CoVs tend to have a restricted host tropism; however, they have a propensity to acquire mutations that can facilitate host “jumping”, or alteration in tissue tropism as seen in the emergence of porcine respiratory virus, the respiratory variant of transmissible gastroenteritis virus [87]. Another genetic tool of CoVs is the ability to recombine, which has been demonstrated by the ability of infectious bronchitis virus isolates to recombine with vaccine strains in the field [87]. Therefore, therapies targeted to RNA viruses must consider the quasispecies nature of the viral population, the ability of the virus to recombine, as well as the rapid ability of RNA viruses to mutate in response to environmental pressure. As the sequence analyses, escape mutant generation and combination testing demonstrate, careful examination of how hmAbs will work together is an important aspect of rational therapeutic cocktail design.

Potential for undesired consequences of passive immunotherapy

Infection with SARS-CoV in humans or immunization of transgenic mice results in the production of mainly non-neutralizing Abs [66,69,79]. A concern with SARS-CoV is the potential for ADE of infection, as seen in FIPV [60,61]. Enhanced hepatitis was found in ferrets previously vaccinated with modified vaccinia Ankara expressing the S protein upon SARS-CoV challenge [88]. In addition, previous studies using several neutralizing hmAbs demonstrated that these hmAbs enhanced infection of pseudotyped virus expressing palm civet S protein in vitro [85]. Interestingly, vaccination with a cDNA that encoded only the ectodomain of SARS-CoV S protein eliminated enhancement of infection of the palm civet pseudotyped virus [85]. It is important to note that the XenoMouse® and the HuMAb-Mouse® were immunized with the ectodomain of S protein [66,67]. Unlike the other hmAbs produced, those generated from XenoMouse® are IgG2 isotype [66]. Advantages of the IgG2 isotype include failure to activate the classical complement pathway and low affinity for FcR on macrophages and other phagocytes. Therefore, IgG2 hmAbs are less likely to facilitate ADE [55].

An additional concern is the possibility of cross reactivity of anti-SARS Abs with self antigens. Serum samples from SARS patients contained Abs that cross-reacted with epitopes on lung epithelial cells and caused cytotoxicity in vitro [89,90]. These Abs reacted predominantly with two defined epitopes within S2 (amino acids 927–937 and 942–951) and not other epitopes within the S protein [89]. Availability of several neutralizing Abs should allow selection for those that do not recognize cross-reactive self-epitopes.

CONCLUSION

Human mAbs are quickly becoming a desirable therapeutic option; since 2009, an additional seven
mAbs have been brought to market, six of which were developed using hmAb technologies (Table 1) [65]. Adalimumab, developed by phage display technology, was the first fully hmAb to be used therapeutically targeting TNF-α to treat a variety of autoimmune disorders [64,65]. Several years later, another hmAb panitumumab, generated using transgenic mice, was approved for the treatment of epidermal growth factor receptor-expressing colorectal cancer [64,65]. The majority of therapeutic mAbs are indicated for the treatment of cancer or a variety of autoimmune diseases [64]. Currently, one humanized mAb has been approved for the treatment of infectious disease (Table 1). Respiratory syncytial virus infections, known to cause significant infant morbidity and mortality, are now successfully prevented with a humanized anti-respiratory syncytial virus Ab (palivizumab) given prophylactically. Palivizumab was generated by inserting the V regions from the mouse mAb into human IgG framework [91]. Rapid clearance, reduced efficacy and adverse reactions seen with chimeric, humanized or Abs from different species may be substantially avoided by using hmAbs [71].

Often neutralizing Abs when used as passive therapy likely “blunt” virus replication, allowing the innate and adaptive immune system sufficient time to mount an immune response [56]. Antibodies have proven important in the response to SARS-CoV [23,43,44,46,73,92,93]. Recovered patients showed a higher Ab titer and neutralizing Abs against S protein were maintained [93]. A limited retrospective study of 40 SARS-CoV patients reported that administration of convalescent patient serum protected against infection, with no adverse events, indicating that passive therapy could be a safe and effective treatment [1,94]. Abs that neutralize in vitro usually can also confer in vivo protection resulting in reduced viral titer [24,44,85,92,93,95]. The hmAbs reviewed here are candidates for passive immunotherapy to provide immediate protection to individuals who may be exposed to SARS-CoV due to re-emergence or laboratory exposure.

The emergence of SARS-CoV resulted in the rapid production of several groups of hmAbs with therapeutic potential, highlighting techniques that may be used to generate hmAbs against other emergent viruses or CoVs currently circulating in bat species. Careful study of SARS-CoV S protein specific hmAbs have contributed to a greater understanding of SARS-CoV infection, highlighting the potential utility of hmAbs not only as immunotherapeutic agents, but also as molecular biology tools.

CONFLICT OF INTEREST

Authors are listed as co-inventors on an Amgen patent without any financial interest.

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