Therapeutic Neutralizing Monoclonal Antibodies: Report of a Summit sponsored by Operation Warp Speed and the National Institutes of Health

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INTRODUCTION

Neutralizing Antibodies for COVID-19 Scientific Summit

In response to the unprecedented coronavirus SARS-CoV-2 pandemic and associated COVID-19 disease, the scientific community is rapidly developing and testing multiple potential approaches for COVID-19 treatment and prevention. One such approach is neutralizing antibodies (nAbs) – antibodies that can bind to SARS-CoV-2 and stop infection. Operation Warp Speed (OWS), in collaboration with the National Institutes of Health (NIH), hosted a virtual scientific summit to explore the current state and future opportunities for nAbs to treat COVID-19. The objectives of the summit were to: 1) review key considerations for assessing nAbs to treat COVID-19 and generate a consolidated knowledge base through development of a white paper; 2) discuss the current state of nAbs and potential areas of optimization for future work; and 3) share key learnings from other diseases and implications for prevention and vaccine development.

The summit included panel discussions on the following five topics:

- Antibody-dependent enhancement
- Epitope binding and viral resistance
- Effector function and antibody optimization
- Lessons from other fields
- Assay standardization

For each topic, this white paper addresses the state of the field, positions on key issues and supporting evidence, immediate lessons for COVID-19 nAb development, long-term implications, and summaries of the summit discussions. This white paper is intended to inform and accelerate development of therapeutic nAbs for COVID-19 through widespread dissemination of expert knowledge and opinions from scientific leaders in the field.

The nAb scientific summit opened with remarks from Dr. Francis Collins, director of the NIH, and Dr. Janet Woodcock, Therapeutic Lead for Operation Warp Speed and director of the Food and Drug Administration (FDA) Center for Drug Evaluation and Research (CDER). The key points addressed in this introduction included:

- Setting the stage for this summit, which is intended to draw upon expertise of the participants and experience from uses of nAbs in other contexts to optimize future applications of nAbs in the treatment of COVID-19.
- Description of convalescent plasma therapy as the prototype of passive immunity, leading to the idea of using monoclonal antibody therapies to treat disease.
- Articulation of the keen interest in exploring the use of convalescent plasma, with numerous ongoing studies. However, many studies are not randomized and/or lack sufficient power, making interpretation of results difficult.
- Sharing preliminary results from exploratory analyses of studies conducted under convalescent plasma Expanded Access Protocols (EAP), which suggest possible benefit for some COVID-19 patients under some conditions. However, prospective analyses and randomized clinical trials are needed to fully understand the effects of this therapy.
- Acknowledgement that our understanding of convalescent plasma and potential antibody therapies is rapidly evolving as data emerge and more rigorous studies and analyses are conducted, and additional information is needed to illuminate the path forward for potential nAb therapies.

SESSION 1

Antibody-dependent enhancement

Presenter:
Barney Graham (VRC/NIAID/NIH)

Moderator:
Ann Arvin (Stanford)

Panelists:
Amanda Peppercorn (GSK/Vir)
Jeffrey Ravetch (Rockefeller)
Mark Esser (AstraZeneca)
Takashi Komatsu (FDA)

Summary of the discussion on the potential for antibody-dependent enhancement

Ann M. Arvin, M.D., Stanford University

Protection by antiviral antibodies involves neutralization through the Fab fragment and immune activation, clearance of viral particles and killing of infected cells through functions of the Fc domain of the immunoglobulin molecule. Antibody-dependent enhancement (ADE) of infection as classically defined refers to virus entry via antibody binding to the virus and Fc receptors on immune cells, which could theoretically alter the host cell tropism and activate these cells to produce inflammatory cytokines. Classical ADE is considered unlikely in the context of antibodies against SARS-Cov-2 because viral replication is abortive in myeloid cells and the potential for mitigation of Fc-mediated activation of complement, macrophages, neutrophils, or natural killer cells by potent neutralizing activity.

Infection with dengue, a flavivirus, provides the only compelling evidence that the mechanisms of antibody-mediated protection have the potential to enhance a human viral disease. Secondary infection with a different dengue serotype is associated with a small increase in the risk of severe disease even though secondary infection is uniformly associated with an ADE signal *in vitro*. Notably, the risk of more severe disease in patients is Fc-mediated, but not by the process measured by ADE assays. Instead, it was associated with changes in antibody binding due to altered IgG glycosylation, which further shows that ADE of disease differs from ADE of infection measured *in vitro*.

ADE of disease is differentiated from the vaccine-associated enhanced respiratory disease (VAERD) that occurred when a formalin-inactivated respiratory syncytial virus (RSV) vaccine was given to children in the 1960s. Although severe RSV disease was associated with antibodies that had poor neutralizing activity but high binding to viral proteins, VAERD is largely a T-cell rather than an antibody related event, reflecting a Th2-biased response. Effects in the two fatal cases were immune complex deposition indicating complement activation and allergic inflammation with eosinophilic and neutrophilic alveolitis, indicating an IL-4 dominant response instead of the interferon-g response to natural RSV infection.

One hypothesis has been that waning of neutralizing antibodies might promote ADE, but such an effect has not been documented over many years of monoclonal antibody prophylaxis against RSV pneumonia in infants. In the context of SARS-CoV-2, the large body of safety data from the clinical experience with COVID-19 convalescent plasma has resolved in large measure questions about the risk of disease from low-affinity or poorly neutralizing antibodies.

In vitro assays do not predict ADE of disease because they do not reproduce the condition of variable Fc receptor expression on different cell types and the release of inflammatory cytokines is the expected functional response of these cells to Fc receptor-mediated signaling. Animal models that can distinguish between the protective benefits of SARS-CoV-2 antibodies and their potential to enhance disease have not yet been developed, in part because of the host range of the virus is limited. A critical obstacle to their design is that Fc receptors are species-specific and only human immunoglobulin Fcs have the capacity to engage human Fc receptors. Animal models that reproduce the human Fc/Fc receptor interaction can be engineered and it is expected that clinical findings of antibody efficacy can be used to establish their predictive value.

Although non-clinical models are recognized as not definitive and should not delay clinical studies, the FDA encourages that such studies be done in parallel with clinical trials. Given that there is much more to learn about COVID-19 disease and that many thousands of patients will be treated, it is useful for pre-clinical safety studies to proceed together with clinical development.

Monoclonal antibodies against SARS-Cov-2 have been designed to extend antibody half-life by introducing YTE or LS mutations into Fc domain; the YTE mutation also reduces Fc receptor binding. The evidence from the use of monoclonal antibodies for HIV infection is that having an intact Fc does not cause disease and that Fc effector functions are important in both the therapeutic and prophylactic settings. These Fc functions contribute to viral clearance and to priming of adaptive responses.

Clinical trials to evaluate monoclonal antibodies for treatment or prophylaxis of viral infections include close monitoring of laboratory tests for safety. While there are a number of biomarkers that detect immune hyperactivity, COVID disease itself has large differences in manifestations, including many immunologic and inflammatory consequences. As a result, no specific biomarker is expected to identify a potential signal for ADE of disease. Immune complex phenomenon might occur when viral antigen levels are high but prior experience with many viral infections and antibody interventions suggests that this is would be rare.

The occurrence of multisystem inflammatory syndrome in children (MIS-C) following SARS-Cov-2 infection is consistent with an antibody-mediated process and responds to high dose immunoglobulin treatment to modulate immune complexes. However, the basis for antibody reactivity in multiple tissues is obscure, which is also the case for Kawasaki's syndrome. While the mechanism is unknown, MIS-C is likely to be a cross-reactive antibody response, not ADE, and is not expected to be disease manifestation that will point to a safety issue.

In summary, to discern unexpected events that may represent potential ADE of disease, clinical studies of monoclonal antibodies against SARS-CoV-2 will need to involve cohorts of sufficient size to detect any unusual or more severe manifestations of disease compared to placebo recipients and evaluate safety in populations with varying severity of disease, viral burden and risk. These studies will be informed by a better understanding of the immunologic abnormalities associated directly with COVID-19 disease and the predisposing factors for severe disease from epidemiologic data.

Antibody Dependent Enhancement

Barney S. Graham, MD, PhD, Vaccine Research Center, NIAID, NIH

Antibodies have distinct functional domains that allow recognition of antigenic surfaces through a variable domain and interaction with other elements of the immune system through the constant domain. The complementarity determining regions are designed to make highly specific interactions determined by topology, chemistry, and accessibility, while the Fc region can interact with the complement system and multiple Fc receptors present on a variety of cell types. The diversity of Fc interactions is amplified by multiple antibody isotypes, subclasses, allotypes, and post-translational modifications that can influence not only effector functions, but also distribution and persistence of the antibody in different tissue compartments. Therefore, the outcome of an antibody interaction with a viral pathogen is determined by many factors including the specific epitopes or antigenic sites targeted on the virus by the Fab region and types of immune elements engaged by the Fc region.

History of enhanced immune-mediated viral disease. For anti-viral passive antibody countermeasures, the goal for treatment and prevention is to block virus infection and rapidly clear virus-infected cells without immunopathology. There is a long history of doing this successfully for infectious diseases with polyclonal or monoclonal antibodies going back to the turn of the 20th century[1] and there have been negligible reports of passive immunotherapy enhancing disease from subsequent infection. However, there are theoretical ways in which antibody could complicate or worsen virus-mediated disease or increase the number of virus-infected cells[2]. There are also examples from certain diseases, particularly dengue, in which secondary infection[3] or infection following partial active immunization[4] tends to increase the frequency of severe disease manifestations. Antigen-naïve children immunized with formalin-inactivated respiratory syncytial virus in alum in the 1960s were not protected from infection during the next winter season. Furthermore, they experienced severe disease characterized by atypical peribronchiolar inflammation with eosinophilic infiltrates and neutrophilic alveolitis. [5,6] One feature of vaccine-associated enhanced respiratory disease (VAERD) was the induction of RSV binding antibody that had poor neutralizing activity leading to evidence of complement activation in the lungs in small airways suggesting immune complex deposition[7]. A similar finding of C4d in airways of young adults who died during the 2009 H1N1 pandemic was associated with high levels of low avidity cross-reactive antibody to hemagglutinin[8]. Immunoprophylaxis or treatment of RSV with polyclonal or monoclonal antibodies has never been associated with enhanced disease or aberrant pathology even when ineffective. New mAbs for RSV prophylaxis are actively being developed[9].

History of immune-modified coronavirus disease. Prior examples of enhanced disease syndromes associated with coronaviruses have only been reported in animals. Feline infectious peritonitis virus (FIPV) is a macrophage tropic coronavirus that causes systemic infection in cats. Passively administered pre-existing sub-neutralizing antibody were associated with immune complex formation, complement activation, and Fc-mediated increased virus replication in macrophages. This caused a necrotizing vasculitis syndrome and mortality in kittens [10,11]. The FIPV vasculitis occurs when antibody is present prior to virus inoculation and it has been shown that FIPV-specific serum antibody levels drop rapidly along with platelet counts early after infection[10]. While FIPV is inherently a macrophage-tropic virus, SARS-CoV-2 and other respiratory coronaviruses are not. In studies of the original SARS-CoV, polyclonal sera containing spike-specific antibody increased entry into FcR-bearing cells, facilitated primarily by FcgammaRII (CD32a and CD32b). This could be shown for both polyclonal serum or RBD-specific monoclonal antibodies using wild-type viruses or SARS-CoV or MERS-CoV spike pseudotyped on VSV or lentivirus reporters and tested in transduced HEK293, Vero, or Raji cells [12,13]. While genome copies of the

virus increased, there was no associated increase in virus replication, and the conclusion was that SARS-CoV has an abortive replication cycle in human macrophages [14].

Enhanced disease has also been associated SARS-CoV challenge after immunization of mice with whole-inactivated [15] or vector-based delivery of wild-type spike[16]. One study reported passive immunization with sera from wild-type spike-expressing recombinant poxvirus vecto[17]r-immunized NHP sensitized recipient NHP resulting in focal lung inflammation post-challenge that was considered severe[16]. While no clinical illness was reported, there was acute lung injury exacerbated by immune sera and associated with inflammatory cytokines attributed to activation of inflammatory macrophages with increased IL-6 and IL-8 and reduced TGF-β production[16]. The pathology occurred in the setting of virus neutralization and reduced virus replication in lungs.

Several commentaries and review papers have recently been published summarizing prior findings in SARS-CoV-1, MERS-CoV, and other respiratory viruses attempting to define the potential risks of treatment or prevention with mAbs and vaccines [17-20]. Others have reviewed the mechanisms antibodies have to interact with coronaviruses [21]. These can be used to guide our discussions.

Unusual features of primary SARS-CoV-2 infection. There are some unusual clinical features of COVID-19 that suggest a systemic inflammatory process that is not seen in other common respiratory virus infections. These include thrombotic events, cardiac inflammation, and neurologic symptoms[22]. There is also a multi-organ inflammatory syndrome of children (MISC) that resembles Kawasaki's disease[23,24]. Because of these extra-pulmonary manifestations of COVID-19 disease, and the findings of immune enhancement in animal models of coronavirus, and because of the urgent need for rapid development of COVID-19 countermeasures, it has been important to intensely scrutinize vaccine, polyclonal and monoclonal antibody safety.

Theoretical safety concerns for monoclonal antibodies. Even though passively-administered antibody has never been reported to enhance virus-mediated disease in humans nor has there been reported immunopotentiation of coronavirus disease in humans, there are theoretical ways that passively administered antibody could enhance disease including 1) increased virus entry and replication in FcR-bearing cells, 2) altered virus tropism through Fc receptors (primarily CD32a or CD32b) or complement receptors (CD35 or CD21), 3) activation of macrophages or other FcR-bearing cells and production of inflammatory cytokines, and 4) production and precipitation of immune complexes in tissues that trigger complement activation and immunopathology. Adverse events from mAb treatment have been almost exclusively related to the mAb interacting with a host cell protein (tissue reactivity) or the immune system reacting to the mAb (anti-drug antibody) with innate or adaptive responses. However, these are all events routinely tested for during the development process of monoclonal antibody products.

Current safety information on COVID-19 and antibodies. To date, there has been no evidence in animal models of SARS-Cov-2 infection of disease enhancement associated with prior vaccination or passive antibody prophylaxis or treatment [25-27]. In addition, more than 5,000 patients with COVID-19 disease have been treated with convalescent plasma without evidence of disease enhancement[28]. SARS-CoV-2 spike monoclonal antibodies have been well tolerated in early phase clinical testing and some have entered phase 3 testing with no reports of unexpected symptoms or inflammatory reactions.

Regulatory and development considerations. There are several potential mAb modifications that could be considered depending on the intended clinical use. For prophylaxis, adding long half-life mutations would allow a single dose to protect through an entire winter season. For treatment, modifying glycans or other elements in the Fc region could alter complement or cell-mediated effector functions. Regulatory authorities will have to make judgements about whether these mAb modifications can be evaluated in

clinical trials before testing unmodified antibodies in humans. Parallel development of animal models to evaluate how passive antibody delivery influences the biology and pathogenesis of SARS-CoV-2 will be important for rapidly adapting to early clinical trial data if needed.

Clinical considerations. Clinical recognition of antibody-modified COVID-19 disease should include both lung and extra-pulmonary observations. In the treatment setting, clinical events would be expected to occur within minutes or hours after mAb delivery. Sudden changes in oxygen requirements, coagulation status including platelet count, or adverse events involving cardiac, renal, central nervous system or integument would be of particular concern.

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SESSION 2

Epitope binding and viral resistance

Presenter
Paul Bieniasz (Rockefeller)

Moderator
David Ho (Columbia)

Panelists
Alina Baum (Regeneron)
Ed Scolnick (Broad)
Katharine Bar (Penn)
MAJ Jeff Kugelman (DoD)

Discussion on Epitopes and Resistance

David Ho, M.D., Columbia University

Following Paul Bieniasz' presentation, there was a lively discussion among the participants, including Alina Baum, Katharine Bar, Jeffrey Kugelman, and David Ho, with the latter serving as the moderator.

SARS-CoV-2 neutralizing monoclonal antibodies reported to date are directed to either RBD or NTD of the spike protein. No one was aware of virus-neutralizing antibodies directed to other regions of the spike protein or to other proteins on the virion surface.

Viral resistance to RBD-directed monoclonal antibodies is now well documented based on the work reported by Regeneron and the Bieniasz Lab. Likewise, viral resistance to NTD-directed neutralizing monoclonal antibodies has also emerged. These observations that demonstrate the ease with which viral escape mutations arise are leading to a level of discomfort in potentially treating SARS-CoV-2 infection with only a single monoclonal antibody, even though a number of companies are currently pursuing the development of a single monoclonal antibody. Numerous comments were made regarding the necessity to conduct careful surveillance for the emergence of antibody-resistant viruses as we move forward with clinical testing of monoclonal antibodies. On the other hand, several other companies are pursuing two-antibody cocktails for both SARS-CoV-2 treatment and prevention. To date it is clearly harder to select for viral resistance using two neutralizing monoclonal antibodies simultaneously. Using HIV treatment as an example, a triple antibody cocktail was also discussed but some thought that might be an overkill. Comments were made that multiple efforts to develop neutralizing antibodies by multiple different companies should continue in order to maximize treatment options in the future. Certain combinations of RBD and NTD monoclonal antibodies have been shown to improve the neutralization profile compared to each individual monoclonal antibody, particularly looking at the IC90 values. Likewise, certain bispecific antibodies have yielded results comparable to a mixture of the two parental antibodies.

A question was posed whether the field should develop a standard of panel of SARS-CoV-2 variant viruses for neutralization by monoclonal antibodies in clinical development. This issue was not specifically answered during this panel discussion but was later addressed during the session on Assay Development and Standardization.

SARS-CoV-2 Monoclonal antibody epitope binding and viral resistance

Paul D. Bieniasz, The Rockefeller University and Howard Hughes Medical Institute

State of the field

Passively administered monoclonal antibodies (mAbs) are among the most promising therapeutic and prophylactic anti-SARS-CoV-2 agents. A number of groups have identified human mAbs that have neutralizing activity against SARS-CoV-2[1-15]. The IC_{50} values of the most potent of these antibodies, measured using *in vitro* neutralization assays, is <10 ng/ml (single to double-digit pM range) and some mAbs have been demonstrated to have therapeutic efficacy *in vivo* using animal model systems. Thus, it is likely that mAbs will be effective in prophylaxis and/or treatment of SARS-CoV-2 infection. Herein, I review issues relating to SARS-CoV-2 mAb epitopes and viral resistance. Given the infancy of this field, some of the following discussion is, by necessity, speculative. However significant progress has been made on several important questions.

Key issues

- What epitopes could or should SARS-CoV-2-targeting monoclonal antibodies be directed at?
- What factors influence the potential for emergence of SARS-CoV-2 mAb resistance?
- What is currently known about SARS-CoV-2 mAb resistance mutations?
- Should mAb cocktails be used and if so, how many antibodies in a cocktail?

Position and supporting evidence

What epitopes could or should SARS-CoV-2-targeting monoclonal antibodies be directed at?

In vivo, antibodies can curtail viral replication principally through (i) neutralization, namely inhibition of infection by binding to virions and/or (ii) mobilization of effector functions such as antibody dependent cytotoxicity (ADCC) that reduce the yield of viral progeny following mAb binding to infected cells. In practice, SARS-CoV-2 mAb discovery campaigns have focused on mAbs that can act by neutralization[1-15], as the role of effector function in the context of SARS-CoV-2 infection is currently not known. Effector functions are typically not measured - these assays are not especially amenable to high throughput, and it unclear whether *in vitro* measurements of ADCC translate to *in vivo* antiviral efficacy. Improvements in screening technologies that enable the evaluation of mAb candidates for effector functions may improve the pipeline for therapeutic antibody discovery. Nevertheless, the ability to neutralize infection *in vitro* is an excellent surrogate for a mAb's ability to bind to conformationally intact, functional trimeric virion spike and is currently the best available predictor of antiviral efficacy *in vivo*, even though mAbs might employ additional mechanisms to curtail viral replication.

The SARS-CoV-2 neutralizing mAbs currently under investigation bind to epitopes on the spike (S) protein, specifically the ACE2 receptor binding domain (RBD, also called S1B) and the N-terminal domain (NTD, also called S1A)[1-15] (Figure 1). Mechanistically, neutralization of SARS-CoV-2 could work in at least 3 ways, which are not mutually exclusive, but could involve distinct antibody binding epitopes: (i) by directly competing with ACE2 for binding sites on the RBD, (ii) by binding elsewhere on the S protein but in so doing occluding S protein access to ACE2 or (iii) by binding to S and preventing conformational changes that are necessary for infection pre or post ACE2 binding. MAb discovery campaigns based on isolation of B-cells that bind to S or to RBD, followed by screening of cloned antibodies for neutralization potency are largely agnostic with respect to neutralization mechanism. Empirically, however, antibodies targeting the

ACE2 binding site on the RBD include those that are the most potent in neutralization assays[1-15]. Some mAbs that bind the RBD close to the ACE2 binding site have neutralization potencies approaching those of the ACE2 binding site mAbs. Antibodies that bind the NTD, or conformational epitopes that include NTD and RBD are somewhat less potent[1-15]. A caveat to this statement is that several discovery efforts have focused exclusively on the RBD-binding antibodies, and it has clearly proven straightforward to discover potent, commonly elicited antibodies targeting this domain. Rarer neutralizing antibodies targeting other epitopes on S, or non-neutralizing antibodies that may have therapeutic activity through effector functions may yet be discovered.

While sequences on the viral spike protein that are conserved among *sarbecoviruses* might be expected to be optimal targets of neutralizing mAbs, the intrinsic conservation of coronavirus sequences (slow drift) means that sequence conservation may reflect the absence of prior selective pressure rather than functional constraints that would indicate attractive mAb targets. The apparently high frequency with which coronaviruses recombine further undermines the supposition that conserved sequences represent optimal targets. Thus, while broadly acting antibodies targeting conserved epitopes or structures may yet be found, discovery efforts should, at this stage, be agnostic with respect to the degree of conservation of the target epitope among *sarbecoviruses*.

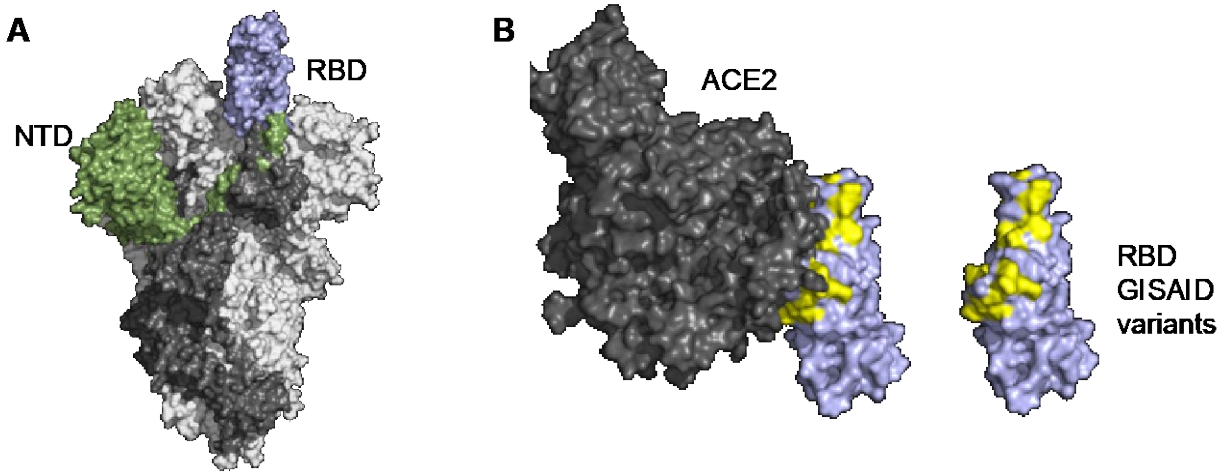

Figure 1 Anatomy of SARS-CoV-2 spike and neutralizing antibody targets. (**A**) Trimeric SARS-CoV-2 spike with one subunit depicted in dark gray with the NTD in green and RBD in blue. The highlighted subunit has RBD in the 'up' (receptor binding) conformation, the remaining RBDs are in the 'down' conformation. (**B**) The RBD depicted in blue bound to ACE2 (grey), or in isolation, with naturally occurring sequence variants in the vicinity of the ACE2 binding site highlighted in yellow.

What factors influence the potential for emergence of SARS-CoV-2 mAb resistance?

With any antiviral therapy, the emergence of resistance is a concern. In the case of SARS-CoV-2, a number of theoretical and real-world parameters will likely affect the frequency with which mAb resistant SARS-CoV-2 variants are generated, selected, and propagated.

SARS-CoV-2 sequence diversity

RNA virus replication is error prone and RNA viruses include some of the most rapidly evolving biological entities. However, intra and inter-individual variation in SARS-CoV-2 sequences is low compared

to many other RNA viruses. The reduced rate of sequence divergence occurs in part because coronaviruses encode a 3'-5' exonuclease 'proof-reading' activity that enhances replication fidelity[16], and perhaps will reduce the rate at which mAb resistance emerges. However, replication fidelity is but one of several variables that affect viral population diversity. An additional key determinant is population size. A single swab from an infected individual can contain 10^9 or more copies of viral RNA[17] and represents a small fraction of the total viral genomes in an individual. Moreover, many millions of individuals have been infected by SARS-CoV-2. It follows that SARS-CoV-2 genomes encoding every possible single amino-acid substitution are present in the global population, and perhaps in a significant fraction of SARS-CoV-2 infected *individuals*. Functional selective pressures such as replicative fitness and immune evasion, as well as the size of transmission bottlenecks will influence the degree to which such variants are maintained or purged from circulating viral populations.

Frequency with which selective pressures are encountered

Given the existence of a globally diverse SARS-CoV-2 population, the prevalence of mutations that confer resistance to therapeutic mAbs will be influenced by the frequency with which circulating SARS-CoV-2 populations encounter those or similar antibodies. The low levels of somatic hypermutation and repetitive manner in which similar, potently neutralizing mAbs have been isolated from convalescent donors (e.g. those based on IGHV3-53[1 18 19]) suggests that potently neutralizing antibodies similar to those whose clinical use is contemplated are readily and frequently elicited. Paradoxically, a significant fraction of COVID19 convalescents, including some from whom potent neutralizing mAbs have been cloned, exhibit low levels of plasma neutralizing activity[1 20 21]. Together, these findings suggest that (i) there is some degree of homogeneity in the neutralizing antibody response to SARS-CoV-2 (ii) natural SARS-CoV-2 infection may often fail to induce sufficient B-cell expansion and maturation to generate high titer neutralizing antibodies, perhaps due to low levels of antigen exposure and viral clearance by other immune mechanisms. Additionally, peak viral burden, which likely corresponds to peak infectiousness and transmission frequency, appears to approximately temporally correspond with the onset of symptoms, and generally occurs before seroconversion[17]. Thus, it is plausible that many, and perhaps most, transmission events involve SARS-CoV-2 variants that are yet to experience antibody-imposed selective pressure in the transmitting individual. Overall, a number of factors will influence the degree to which SARS-CoV-2 populations have been previously exposed to selection by antibodies that are similar to the candidate therapeutic mAbs, and thus the prevalence of pre-existing mAb resistance mutations.

How SARS-CoV-2 mAbs are deployed

How SARS-CoV-2 mAbs are used clinically directly affects the size of the SARS-CoV-2 populations that they will encounter and will directly impact the probability of selecting resistant mutants. The number of viral variants that are transferred during SARS-CoV-2 transmission from one person to another is unknown, but it is likely to be small, and may theoretically be as low as a single infectious unit. Thus, if mAbs are employed exclusively in a prophylactic setting (which may or may not be practical) the probability of selecting mAb resistant variants is reduced. Conversely, if antibodies are given as therapy, then the number of viral variants subjected to mAb selection is much larger. However, even if deployed as prophylaxis, there is a risk that mAbs would inadvertently be administered to individuals who are already infected. In either case, encounters between mAbs and large populations of SARS-CoV-2 increases the probability of selecting of mAb-resistant SARS-CoV-2 variants. The consequences of such selection in individuals, both for the clinical outcomes of the treated individual and for the potential initiation of mAb-

resistant virus transmission chains is currently unclear. The risk of propagation of mAb resistant strains in the general population would clearly be mitigated if their use was confined to hospitalized or otherwise quarantined patients.

What is currently known about SARS-CoV-2 mAb resistance mutations?

The study of SARS-CoV-2 mAb resistance is currently in its infancy. To overcome difficulties inherent in studying mAb resistance in the context of SARS-CoV-2, three groups (our own[22], the Regeneron group[23] and the Whelan group, personal communication) have developed replication-competent chimeric viruses based on vesicular stomatitis virus (VSV) that encode the SARS-CoV-2 S protein (rVSV/SARS-CoV-2). These chimeric viruses have the following features: (1) they replicate rapidly and to high titers, (2) they mimic the SARS-CoV-2 requirement for ACE-2 as a receptor, (3) they are neutralized by convalescent plasma and mAbs with similar potency to authentic SARS-CoV-2, (4) the absence of proof-reading activity in the viral polymerase (VSV-L) results in the generation of virus populations with greater diversity than SARS-CoV-2, for an equivalent viral population size. These features dramatically facilitate *in vitro* experiments to identify functional S protein variants that escape antibody neutralization. Additionally, pseudotyped virus assays based on HIV-1 or VSV particles can rapidly and effectively determine the susceptibility of S variants to mAb neutralization.

Targeting the ACE2 binding site on the RBD with mAbs offers the potential advantage that sequences required for ACE2 engagement may be functionally constrained, potentially increasing genetic barriers to mAb resistance. However, using rVSV/SARS-CoV-2 chimeras, all three groups have readily been able to select resistance to a variety of mAbs, including those that directly target the ACE2 binding site on the RBD (Figure 2). Thus, the RBD surface that binds ACE2 does not appear to be sufficiently functionally constrained that targeting this surface offers obvious advantages in terms of a genetic barrier to mAb resistance. A caveat to this assertion is that the functionality of these mAb-resistant S mutants has been examined in VSV/SARS-CoV-2 chimeras rather than authentic virus. An important remaining question is whether antibody resistant variants exhibit pathogenesis and transmission deficits compared to currently dominant circulating SARS-CoV-2 variants. The presence of mAb-resistance mutations in global SARS-CoV-2 populations be monitored using initiatives such as GISAID (https://www.gisaid.org) and CoV-GLUE(http://cov-glue.cvr.gla.ac.uk/#/home), which at the time of writing contain ~80,000 near full length SARS-CoV-2 sequences. Notably, while the existence of these sequence databases is invaluable for identifying potential mAb resistance mutations, improvements in sequence availability and curation, as well as high quality analyses of intra-patient variation will undoubtedly facilitate prognostication and monitoring of resistance mutation prevalence. Currently, sequence databases are dominated by consensus sequences derived from viral populations in individual patients. Nevertheless, mutations in and around the ACE2 binding site, including those demonstrated to confer resistance to neutralizing mAbs, are clearly present in circulating SARS-CoV-2 natural populations, albeit at low frequencies, consistent with the absence of profound genetic barriers to mAb resistance.

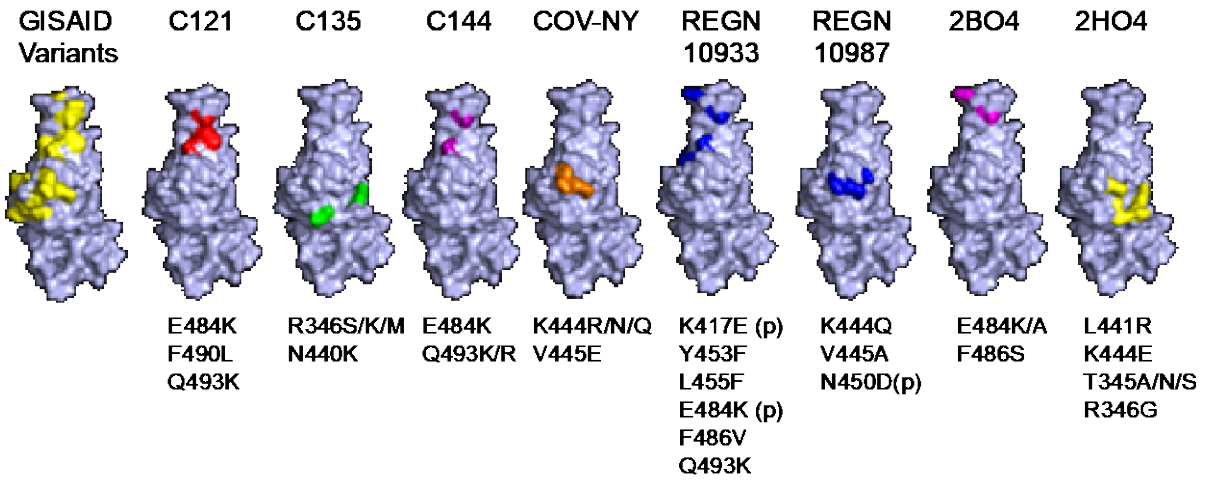

Figure 2 Examples of resistance mutations selected by SARS-CoV-2 neutralizing antibodies targeting the RBD, using rVSV/SARS-CoV-2. Leftmost figure indicates (in yellow) positions in the vicinity of the ACE2 binding site on the RBD where variation has been documented in circulating SARS-CoV-2 populations. Remaining figures indicate resistance mutations selected using monoclonal antibodies (except COV-NY, where selection was done using convalescent plasma). Data from https://www.gisaid.org, references [22] [23] [24] and S. Whelan and Ali Ellebedy (personal communication).

Should mAb cocktails be used and if so, how many antibodies in a cocktail?

A key consideration of the use of any antiviral therapeutic approach is that cocktails often elevate the genetic barrier to resistance to the applied therapy. Using the rVSV/SARS-CoV-2 approach, our own and the Regeneron group have shown that under *in vitro* conditions, where it is straightforward to generate single mAb-resistant S variants, the emergence of resistance can be effectively suppressed through the use of two potent RBD-specific monoclonal antibodies whose resistance mutations are non-overlapping[22] [23]. Given that single amino acid substitutions appear sufficient to generate resistance to single antibodies, then *N* mutations are required to generate resistance to a cocktail of *N* antibodies. If (for example) mutations conferring resistance to a single mAb exist in a viral population at a frequency of ~10^{-5}, then mutants that can resist a two-mAb cocktail are expected to be present at a frequency of ~10^{-10}. (This assertion pre-supposes that mAbs in a given two mAb cocktail have been selected so as to select non-overlapping resistant mutants, and that there is no functional interaction between the resistance mutations selected by each antibody).

A second argument in favor of the use of antibody cocktails is the potential for *in vivo* potentiation. Combinations of neutralizing mAbs do not appear to act synergistically during *in vitro* neutralization. However, given the greater number of potential mechanisms by which antibodies curtail virus replication *in vivo* there is potential for synergy that is not evident with *in vitro* neutralization assays. For example, mAbs that work primarily by neutralization, might synergize with those that work primarily via mobilization of ADCC. The larger impacts on viral population size that may result from synergistic actions of antibody cocktail components might have additional benefits curtailing the emergence and propagation of mAb-resistant mutants. However, the benefits of combining 2 or more antibodies in a therapeutic cocktail must be weighed against manufacturing, pharmacologic and other considerations that complicate the development of multi-component therapies. Bi-specific antibodies (antibodies whose two Fab domains recognize distinct epitopes) offer one possible route to targeting multiple epitopes, and

thereby elevating genetic barriers to resistance, using single agents. However, it is likely that in many cases, losses of avidity and activity that would ensue following mutations that cause loss of recognition by one of the two antibody arms.

Immediate lessons for mAb development

Based on the above considerations, a number of conclusions can be drawn and/or recommendations given:

(i) Antibodies that target the RBD have been shown to be the most potent in *in vitro* neutralization assays. A number of highly potent (low ng/ml IC_{50}) RBD-specific mAbs that target sequences within or proximal to the ACE2 binding site in the RBD are highly promising for therapeutic or prophylactic applications. NTD-specific mAbs are less potent in *in vitro* neutralization assays. Non-neutralizing antibodies that have *in vivo* antiviral activity have yet been identified.

(ii) The genetic barrier to the development of resistance to SARS-CoV-2 neutralizing mAbs appears low. Indeed, all documented attempts to generate VSV/SARS-CoV-2 variants that are resistant to single mAbs have been successful, even those that target the ACE2 binding site within the RBD. While resistance to mAbs targeting the NTD has not been reported at the time of writing, it is very likely that resistance to such antibodies will be equivalently easy to achieve. Indeed, NTD mutants that confer resistance to antibodies present in convalescent plasma have been isolated[22]. Moreover, sequence variants are present at low frequency in naturally circulating SARS-CoV-2 populations that are demonstrated or predicted to confer resistance to mAbs. For these reasons, it would appear prudent to deploy therapeutic mAb cocktails with at least 2 mAbs with non-overlapping epitopes.

(iii) Neutralizing antibodies entering clinical trials should ideally be examined for the spectrum of mutations that confer resistance. Those that are chosen for inclusion in cocktails should target epitopes that have non-overlapping resistance profiles. Alternatively, antibodies that are combined in cocktails should target physically distinct epitopes as demonstrated by clear lack of competition (simultaneous binding) in ELISA or biosensor assays. In some instances, mAbs that target the same epitope, but have differential tolerance for individual mutations may be combined.

(iv) Resistance mutants for mAbs can be straightforwardly identified. Animal model studies and human clinical trials of mAbs should include a resistance monitoring component[25]. In the context of mAb treatment of already infected individuals, close monitoring of viral sequences should be initiated at the time of therapy initiation and frequently thereafter. Similarly, in prophylaxis trials, sequence analysis of any infections that occur should be undertaken.

Long term implications

The potential for the long-term utility of mAbs to be blunted by the emergence of resistant viral variants is enhanced because antibodies that are elicited during infection, by vaccination, or administered as convalescent plasma and therapeutic mAbs can all target overlapping determinants. It would be prudent to research and monitor the impact of all of these factors on the emergence of SARS-CoV-2 mutations that confer antibody resistance. Indeed, if over the long term, mAbs become an important tool in the control of SARS-CoV-2, it may become important for epidemiologists to routinely track the prevalence of antibody-resistant SARS-CoV-2 variants, in much the same way as drug resistance in HIV-1 and influenza, as well as antibiotic resistance in bacteria is tracked. Some of the questions pertinent to the use of mAbs that could be addressed in future studies include:

(i) What is the "polyclonality" of the neutralizing antibody response in natural infection and following vaccination? How does the specificity of natural or vaccine (e.g conformationally stabilized S proteins, or isolated RBD immunogens) elicited protective antibodies overlap with the specificities of mAbs entering the clinic as prophylactics/therapeutics?

(ii) Millions of individuals have been infected with SARS-CoV-2 and among them, neutralizing antibody titers are extremely variable[1 20 21]. Those with weak immune responses or waning immunity could become re-infected at some unknown frequency. Indeed, there have been several recently documented cases of SARS-CoV-2 re-infection. Will waning immunity following natural infection or vaccination increase the frequency of encounters between diverse SARS-CoV-2 populations and neutralizing antibodies and drive the emergence of antibody resistant variants?

(iii) Will convalescent plasma therapy drive the emergence of neutralizing mAb-resistant SARS-CoV-2 variants? Many individuals have received, and likely will receive sub-optimal levels of human antibodies in the form of plasma. Obviously, some of the antibodies in plasma have the same or similar specificity as the therapeutic mAbs in clinical development.

(iv) While mAb-resistant S variants are readily generated using VSV/SARS-CoV-2 in the laboratory and are clearly functional, do mAb-resistant S variants have reduced fitness or transmission in a natural setting?

(v) It will likely be necessary to isolate subsequent generation mAbs if resistance to the mAbs currently in development renders them obsolete. Will it be possible to isolate 'broadly neutralizing' antibodies that neutralize a range of pandemic-threat coronaviruses? MAbs that neutralize both SARS-CoV-2 and SARS-CoV have been discovered, but there are typically large discrepancies in the potency with which the two viruses are neutralized[11]. The development of bispecific antibodies has the potential to improve potency and breadth.

In conclusion, the SARS-CoV-2 pandemic presents unique challenges and opportunities for the deployment of prophylactic and therapeutic monoclonal antibodies. Clearly, antibody epitope selection and resistance may impact the utility of mAb therapy and will requires investigation and monitoring in the months and years to come.

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SESSION 3

Effector function and antibody optimization

Presenter
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Moderator
Michel Nussenzweig (Rockefeller)

Panelists
Christos Kyratsous (Regeneron)
Dan Barouch (Harvard)
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Discussion Session 3: Efficacy – Effector function and antibody optimization

Michel Nussenzweig, Rockefeller University

- Presentation
    - Fc mediates numerous innate immune functions, can destroy anything to which an Ab is bound. In a simplistic view - Fab: specificity, blocking, neutralizing; Fc: longevity. In reality - Fc domain links innate and adaptive immune system.
    - Numerous signals that implicate Fc function in COVID:
        1) Polyclonal Ab signals (convalescent plasma) – protection associated with Ab titer, but not necessarily neutralization ability
        2) mAbs – in hamsters: potent neutralizing but virus/disease not completely eliminated
        3) other Coronaviruses: passive transfer of antibodies protective; Fc function important
        4) Vaccines – might be dependent on tissue type; emerging vaccines point to important functions for resolution in nose vs. lung
    - Need to balance Fc domain benefits vs potential but unproven liabilities.
    - Opportunities for Fc engineering – not simply on/off. Control half-life while retaining functions; changes to functions; improve stability; mutations to improve manufacturability.
    - Fear of ADE has somewhat paralyzed development, but toolkit is quite elegant.
- Other isotypes? Need more experimentation. Some literature of IgA responses, there are correlates with protection. Conceptually it makes sense IgA on mucosal surfaces should be robust against challenges. However, there needs to be careful experimentation to test. Not entirely clear where protection occurs (at mucosal surface or in tissues underneath). Ex. in HIV there is protection at mucosal surface but most protection occurs systemically from clearance of viral reservoirs.
- Have been thinking about IgA/IgM not just for location but for their avidity. Also viral clustering and role of immune system in clearing clusters – has anyone looked at that?
- We have a lot to learn about IgA, complicated molecule. Technologies not as advanced as they are for IgG. Have to look at the end game – can you manufacture at scale in timeframe needed? Have to leverage existing platforms for manufacturing. IgG (especially IgG1) platforms already there.
- Are we looking at reduction of nasal shedding? This may be important for transmission.
- A year from now, when we have vaccines, how would we use mAbs? There will be a lot of people who don't respond to vaccines, especially people in nursing homes → mAbs could be useful here. There are also important clinical applications like rapid protection for high-risk groups. Given to populations within a targeted subgroup (household, meat packing plant, etc.). mAbs also have potential uses in therapy for individuals that did not respond to vaccine or to whom vaccine was not administered.
- Do we know anything about different epitopes, their ability to protect/neutralize? Epitopes matter in cocktails because you don't want them to compete. Want to hit different epitopes both neutralizing. Gives benefit for prevention of escape.
- Through all sessions today – need high-throughput screening. Need various platforms, like HIV, ability to test efficacy.
- You can get different germ line responses to different epitopes

- Epitope specificity is very under-appreciated – the work done for flu really show the importance of epitope specificity – Ebola as well. Thinking of the molecule as a 'holistic' platform will be critically important to designing cocktails.
- Problems/advantages of bispecific Abs? Often see similar clearance rates for Abs in cocktails. Very rare to have Abs in a cocktail to clear at different rates. Make sure your bivalent antibody binds to your epitopes. You might have one arm bind and one "dummy" arm. Not as many advantages to bispecific compared to cocktail. Unless you have evidence that cross-linking antibodies confers advantage. Manufacturing not an issue for bivalent vs. bispecific. Bispecifics show some advantages in vitro, it is one Ab instead of two so may have cost savings.
- How do you balance tuning the Fcs for advantages vs. introducing safety problems? In cancer, where you don't have much of a choice, there is an impetus to use this type of enhancement. In infectious disease this is more complicated, especially. in uninfected individuals (i.e. prophylaxis.). Have to consider off-target effects. Bar is much higher.
- Vaccines introduce heterogeneity of Fc, differential binding to different Fc receptors. Same risk of enhancing disease as vaccine. All the mAb technologies we're talking about are to reintroduce the heterogeneity. Heterogeneity means we don't limit ourselves to only a single response; my opinion is risk is minimal in contrast to loss of opportunity.
- Does FDA agree (w/benefits vs. risks of heterogeneity)? In cancer, they do, we have all variants. In infectious disease we have only a few examples.
- ACTIV/OWS have set up programs for Abs for therapy but another area is prevention. Has NIH been thinking about program like ACTIV for prevention? Trials are already underway, not part of ACTIV. Focused on very high-risk individuals. These trials are more challenging to execute compared to treatments. Have to "parachute in" when outbreak starts. Studies planned or underway.

Tuning the function of monoclonal therapeutics to SARS-CoV-2

Galit Alter, PhD. Ragon Institute of MGH, MIT, and Harvard

Given the rapid spread of SARS-CoV-2, therapeutics are urgently needed to not only prevent but also treat COVID-19. Among the strategies, passive transfer of monoclonal antibodies, which are able to both drive directed antiviral activity and also tune the immune system, represent an ideal class of therapeutics, potentially suited for both prevention and therapy. However, emerging data pointing to the possibility of antibody-mediated enhancement of disease following vaccination against SARS-CoV-1[1] and monoclonal therapeutic treatment of SARS-CoV-2 infected hamsters[2] have raised the importance of carefully considering the role of the antibody fragment crystallizable region (Fc) in SARS-CoV-2 therapeutic design. While the antigen-binding domain (Fab) is a critical determinant of antigen-specificity and binding affinity, the constant-domain (Fc) interacts with the immune system offering opportunities to drive enhanced half-life, immune clearance, and inflammation. Whether the Fc-domain is a permanent liability or whether selective Fc-engineering could maximize clinical benefit –differentially for prophylaxis or therapeutics – is the subject of this white paper.

Polyclonal antibody signals: Human convalescent plasma is currently being explored widely to treat and prevent COVID-19 and large randomized controlled clinical trials (RCT) are underway. Smaller RCTs, matched- control studies, and case series studies have provided mixed results, presumably due lack of appropriate statistical power[3,4] but a recent meta-analysis, although not peer-reviewed, combining data of 12 studies suggests a positive efficacy signal of reduced mortality in convalescent plasma treated individuals[5]. Although it has been assumed that neutralizing antibody titers are the therapeutic correlate, it remains to be determined if other mechanisms, ie. Fc-functional properties of anti-SARS-CoV-2 antibodies or other specific anti-inflammatory factors in human plasma might play a role.

Monoclonal signals: A tidal wave of monoclonal therapeutics have emerged[2,6-10]. Two critical insights have been garnered from in vivo experiments thus far with these therapeutics: 1) treatment of hamsters – the pathological model of SARS-CoV-2- results in attenuated but not complete reversal of infection; and 2) at low antibody concentrations, enhanced weight loss was observed, potentially pointing to disease enhancement at sub-neutralizing antibody doses[2]. These data suggest that even the most potent neutralizing antibodies, administered at relatively high doses, will only contribute to partial control of infection, if they depend on Fab-activities alone, and that wildtype IgGs at low concentrations may drive inflammatory responses. Yet, Fc-engineering linked to enhanced knowledge of protective immune correlates, could lead to strategic rational monoclonal design able to confer protective immunity in the absence of disease liabilities. However, it is plausible that the functions required for protection may be distinct to those required for treatment.

Hints from other Coronaviruses: Antibodies represent the primary correlate of immunity following most clinically approved vaccines[11,12]. For most viruses, antibodies able to block infection, neutralize, represent the dominant mechanism of antibody action. However, emerging evidence point to the critical role of additional functions of antibodies in protection against viruses. For example, in the context of Influenza based immunity, neutralization explains some, but not all protection[13]. Subjects with high levels of neutralizing antibodies are not always protected, and monoclonal antibodies that lack neutralizing capacity can also provide protection against influenza in animal models[14]. Likewise, in the setting of other Coronaviruses (CoVs), monoclonal antibodies (mAb) against SARS-CoV-1 also provide protection in mice[15]

and both neutralizing and non-neutralizing antibodies against the Middle Eastern Respiratory Syndrome (MERS) virus afford protection[16-18]. Likewise, both neutralization and antibody dependent cellular cytotoxicity (ADCC) have been linked to protection in SARS infected individuals[19] and animal models [20]. Moreover, vaccine induced humoral immune responses able to drive phagocytosis and complement activation, but interestingly not ADCC, in collaboration with neutralization, have been linked to protection against SARS-CoV-2 challenge[21]. Given the remarkable infectiousness of SARS-CoV-2, with an estimated R_0~2.5[22-26], strategies to provide maximal protection from infection and disease may require both blocking and post-infection eliminating-antibody functions for maximal immunity.

Signals from vaccines: While several SARS-CoV-2 vaccine candidates drive high levels of neutralizing antibodies, these functional antibodies do not offer complete protection from viremia in the upper respiratory tract despite challenge close to peak immunogenicity antibody titers[21,27,28]. Whether this is related to the difficulty in maintaining high levels of neutralizing antibody titers in the upper respiratory tract or whether the more limited immune effector cells present in the upper respiratory tract that may be mechanistically essential for full humoral immune control/clearance of the virus are causal, remains unclear. However, antiviral control mechanisms differ considerably across the upper and lower respiratory tract. Differences in mucus, cellular infiltrates, and temperature in the upper respiratory tract have all been linked to differential pathogen infectivity and evasion. While the lower respiratory tract is persistently patrolled by cells of the innate and adaptive immune system, lower cellularity in the upper respiratory tract renders surveillance more difficult[29]. Thus, antibodies may have the capacity to leverage far more antiviral functions in the lung, compared to the upper respiratory tract, resulting in nearly an exclusive dependence on Fab at the portal of entry. However, increasing data points to opportunities to engineer antibodies to trap pathogens in mucus, to leverage complement, or even to recruit non-canonical functions in less-cellular environments. Thus exploring design strategies – even including the simple usage of IgA isotypes, local administration, or sustained release of antibodies – to enhance bioavailability and bio-activity of monoclonal therapeutics in the upper respiratory tract may translate to enhanced restriction at the portal of entry.

Signals from natural infection: Emerging data show that individuals that recover from mild SARS-CoV-2 infection develop low levels of neutralizing antibodies, while individuals with severe disease and the elderly[10,30] often generate robust neutralization, calling a role for neutralization for resolution of natural infection into question. Thus, while it is clear that neutralization is critical for initial viral restriction, it is unclear whether neutralization is required later for disease clearance. Distinct immune control mechanisms are likely to be key to control and clearance of viruses that escape initial restriction by neutralizing antibodies. Instead the clearance of the virus via opsinophagocytic mechanisms and destruction of the virus and infected-cells may be more critical for overall resolution of infection. The experiments to test this hypothesis, are underway, with SARS-CoV-2 monoclonal antibodies, with different Fc-functions, having entered clinical testing[31,32]. While these therapeutics will be used in populations with slightly different clinical phenotypes, the ultimate question of whether Fc-function is required for viral containment and clearance will be defined. However, it is critical to note that additional modifications could be exploited to selectively enhance complement, but not NK cells or phagocytosis. These highly specialized point mutations offer enhanced control over inflammatory mechanisms, and could be tailored by severity, timing from infection, or population (children, elderly, etc) to drive custom-precision level control over disease.

Fc-engineering: Emerging data point to a critical protective role for Fc-effector function in protection against several infectious diseases, including HIV, malaria, tuberculosis, influenza, Ebola virus, and beyond[33-38]. However, the precise functions involved in protection differ across pathogens and even across antigens/epitopes within a pathogen-specific response[39]. Two modifications of the Fc-domain are naturally explored by our immune system to tune antibody effector function[40]: 1) alteration of the isotype/subclass of the Fc-domain and 2) changes in IgG Fc-glycosylation. Together the combinatorial diversity of these 2 modifications give rise to hundreds of distinct Fc-domains during a natural immune response, that alter antibody affinity for Fc receptors, complement, and lectin-like receptors found on all immune cells[41]. Both modifications have been explored broadly in the monoclonal therapeutics community, giving rise to opportunities to differentially harness specific immune effector functions. For example, IgG1 Fc-point mutations have shown promise in the treatment of HIV infection[42,43] as has afucosylation for Ebola treatment[44], both modifications that enhanced binding to FcγR3a present on Natural Killer (NK) cells. Conversely, several modifications have been developed to turn off Fc-effector function to avoid potential deleterious activation of the innate immune system[41]. Dozens of IgG1 point-mutations have been described that enhanced antibody dependent cellular cytotoxicity (ADCC), antibody dependent phagocytosis (ADCP), and antibody dependent complement (ADC)[40,41]. Moreover, mammalian cell lines, engineered plants, and chemo-enzymatic approaches exist to control IgG1 Fc-glycosylation to shape ADCC -via the removal of fucose or additional of a bisecting N-acetyl-glucosamine- or to reduced inflammation -via the addition of sialylation. Additional point mutations have been defined that alter antibody half-life (YTE, LS, etc.), via improved binding to the neonatal Fc-receptor (FcRn), involved in antibody recycling, either by reducing off rates (YTE and LS), by increasing affinity within the endosome with a pH of 6.5 (LSF, C6A), or by increasing initial pH7 FcRn affinity (YTE). However, novel mutations and strategies to alter glycosylation for IgG1, additional subclasses and isotypes, continue to emerge, providing an unprecedented opportunity to specifically and selectively tune antibody effector function. Moreover, bi-specific antibodies can also be generated linking multiple epitopes or linking effector cells to the pathogen (killer T cells) offering additional opportunities to engage a broader landscape of pathogen-targets or drive immune effector functions, respectively. However, to date, monoclonal antibody engineering for SARS-CoV-2 has focused nearly exclusively on simple modifications (Fc-on, Fc-off, wild-type), due to our poor understanding of the humoral functional correlates of immunity that track with protection or convalescence. The identification of the precise antigenic-targets and linked functional immune mechanisms involved in pathogen containment/clearance will enable the rational design of therapeutics able to harness and direct precision level protection against this unpredictable pathogen.

Manufacturability: While modifications of the Fc to control immune-mediated response may be used to engineer antibody-based ~~bio~~therapeutics, the Fab of an individual antibody is responsible for epitope targeting and specificity. The natural B-cell response to an invading pathogen includes genomic recombination with mutations at the junctions, as well as somatic hypermutation during B-cell division driven by positive epitope engagement. The mutations which occur by these processes are throughout the variable domain, not limited to just the CDRs, and drive affinity and specificity, support CDR loop orientation for optimal epitope engagement, and both core Ig fold and Fab interface stability. However, B-cells in the human body have no selective pressure to produce antibodies optimized for ~~bio~~therapeutic process development and manufacturing. Stresses introduced during development include incubation with media components, filtering, purification, low pH viral inactivation, buffer exchanges, high

concentrations, and freeze/thaw cycles, as well as the realization of sequence liabilities to function such as free cysteines, isomerization and deamidation sites, and oxidation sites. While most mutations from germline are necessary for antibody engagement of target, many may be modified to improve molecular stability, thus improving behavior during development and manufacturing. In the consideration of development timelines, however, when the speed of development does not allow for Fab optimization engineering, computational evaluation methods may be utilized to eliminate antibodies which may pose a high development risk, resulting in biotherapeutic candidates with the highest chance of development success. These selected Fabs may then be placed on optimal constant domains, including modified Fcs, in a final biotherapeutic. However, while IgG1 and a handful of IgG1 Fc-mutants have been established for larger scale GMP manufacturing, emerging next-generation engineered Fab/Fc therapeutics have not been established for GMP manufacturing and thus scalability remains a challenge.

Collectively, the days of turning the Fc- "on or off" are gone. We need not to fear the Fc, but rather take advantage of our emerging skills to master its control over the immune system, and select and design antibodies with the highest probability of manufacturing and clinical success. It is likely that a single "one size fits all" solution may not exist for therapeutics aimed at treating the elderly/children, in prophylaxis vs therapy, or even in alleviating moderate vs severe disease. However, taking advantage of our ability to rapidly adapt therapeutics to maximally engineer protection and production in a targeted manner dependent on the body compartment/tissue, non-pathological immune targets, as well as unique patient population offers an opportunity to push precision therapeutic design into a new era as highly effective countermeasures against SARS-CoV-2 and beyond.

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SESSION 4

Lessons from other fields

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Discussion Session 4: Efficacy – Learnings from other fields

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Important lessons can be gleaned from the use of nAbs in other viral infections:

- RSV: animal models can predict efficacy in humans; nAb can protect against RSV in vulnerable human populations while treatment with nAb in established infection may not be successful; improvements in neutralization may lead to better protection; potent nAbs can help guide vaccine development; nAbs against multiple pneumoviruses have been described (pan-virus family Abs), suggesting that pan-coronavirus approaches should be considered.
- HIV: many potent broadly neutralizing Abs (bnAbs) have been isolated; cocktails of bnAbs may permit extended periods off of ART and may contribute to cure strategies; bnAbs may contribute to approaches to develop an HIV vaccine.
- Influenza: anti-stem nAbs have not shown clinical benefit in trials.
- Ebola: Ab therapy can change the disease course in an explosively replicating, systemically spreading virus; monotherapy has had similar outcomes as compared with a three Ab cocktail.

Key points:

- Despite the lessons learned from the use of nAbs in other viral infections, nAbs and viruses are very diverse. No two Abs/viruses behave in a predictably identical fashion, so extrapolation to SARS-CoV-2 infection is challenging.
- nAb potency is likely a key factor for efficacy against SARS-CoV-2. The most potent antibodies against SARS-CoV-2 bind to RBDs, acting like small molecules blocking RBD binding. Other factors that may influence efficacy include:
    - the breadth of Ab neutralization;
    - effector function;
    - ensuring that adequate nAb levels are achieved at the sites where they are needed; and
    - the therapeutic window for nAb administration, as timing of therapy after infection may be a critical factor for optimizing outcomes.
- SARS-CoV-2 nAbs can contribute to vaccine development by informing which epitopes elicit neutralizing antibodies. For example, potent Abs to SARS-CoV2 target the ACE2 binding site. However, if breadth of coverage is desired then different targets may be required to balance potency and breadth. nAb development may inform appropriate nAb titers and effector functions needed for protection and may serve as benchmarks for vaccine trials. The COVID Prevention Network plans to evaluate biomarkers after vaccination in order to provide a rapid and thorough evaluation of SARS-CoV-2 vaccines.
- Although mAb114 (mono-antibody therapy) has been demonstrated to be effective against Ebola, it is not known whether monotherapy might be adequate for SARS-CoV-2.
  Since a cocktail of nAbs may be needed against SARS-CoV-2, research should focus on more than one nAb clonotype. A regulatory framework that allows for swapping of nAbs in a cocktail could expedite development of new, effective mAb combinations that might be required during the evolution of the SARS-CoV-2 pandemic.

Neutralizing antibodies and SARS-CoV-2: lessons from other fields

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Background

An important early point to make for SARS-CoV-2 in relation to observations on viruses and neutralizing monoclonal antibodies (hereafter "nAbs") from other fields is that, although many lessons can be learned, *no two antibodies and no two viruses behave predictably in identical fashion*. As a single antibody example, there is generally a good correlation between the ability of an antibody to neutralize HIV and ability to kill HIV-infected cells by antibody-dependent cellular cytotoxicity (ADCC) in a much-used assay [1]. Nevertheless, there are cases where two nAbs directed to the same epitope on the HIV envelope spike with similar neutralizing potency and of the same isotype mediate very different levels of ADCC, presumably because they have somewhat different angles of approach to their target and then interact differently with Fc receptors. This functional difference may or may not be reflected in activity in vivo but emphasizes the difficulties in extrapolating between seemingly similar antibodies. Extrapolating between different viruses is equally or more difficult. Nevertheless, observations useful in planning for deployment of nAbs against SARS-CoV-2 can be made when considering other systems and some of these are summarized here.

How is neutralization defined and how do nAbs act anti-virally in vivo? The overwhelming majority of antiviral antibodies in clinical development are neutralizing [2] (Table 1), but this does not necessarily mean that neutralization per se is the sole or dominant anti-viral activity in vivo. One definition of neutralization is "the loss of infectivity which ensues when antibody molecule(s) bind to a virus particle, and usually occurs without the involvement of any other agency" [3]. "Without the involvement of any other agency" (although rarely is complement included) indicates that neutralization is typically measured by simply incubating antibody, virus and target cell together and demonstrating reduced infection. A second definition is "the reduction in viral infectivity by the binding of antibodies to the surface of viral particles (virions), thereby blocking a step in the viral replication cycle that precedes virally encoded transcription or synthesis" [4]. Considering the first definition, the anti-free-virus activity of nAbs in vivo, in contrast to in vitro, may well include "other agencies" such as complement or Ab-binding effector cells (Figure 1). Furthermore, nAbs target functional molecules on the virus surface to prevent target cell entry and these same molecules are typically also presented on the surface of infected cells giving nAbs an opportunity to eliminate such cells via antibody effector functions. Overall, nAbs can provide prophylactic and therapeutic activities in vivo by targeting free virus as well as virally infected cells by a range of mechanisms that include direct blocking of viral entry (neutralization), but also those involving antibody effector functions and less obvious mechanisms such as trapping of virus on Fc receptor-bearing cells and preventing egress of virus from infected cells (Figure 1). The more that is understood about how a given nAb provides anti-viral activity, preferably in vivo, the more effectively can the antibody be deployed for clinical use. A rough rule of thumb is that the more potent a nAb, the more effective it will be anti-virally in vivo, although this rule can be broken if some of the other mechanisms described above are crucial e.g. if antibody effector function is important then nAb angle of approach could determine effectiveness for two Abs that otherwise had similar neutralizing potency as above.

How different are viruses in their responses to nAbs? Clearly, viruses are very different from one another in many respects and these differences can have profound effects on what might be required of a nAb in

the clinic. NAb-mediated protection against HIV, because of the ability of the virus to establish a latent state, may require sterilizing immunity. For other viruses, including SARS-CoV-2, it may be sufficient for a nAb to blunt viral replication sufficiently for innate or cellular immunity to resolve infection without major disease symptoms. Such differences have significance for the likely effective dose of antibody required for protection. In terms of the use of nAbs for therapy, the huge diversity of HIV present in an infected person after even a relatively short period of infection indicates that cocktails of even the most potent and broadly neutralizing antibodies are likely required. For acute viruses such as SARS-CoV-2, for which such diversity is not present, the requirement for nAb cocktails may be less severe. In both prophylaxis and therapy, the role of nAb effector function can vary widely [5-11].

Non-neutralizing antibodies. Finally, non-neutralizing antibodies (nnAbs) can provide protection and act therapeutically against a number of viruses but generally the activity is much weaker than that of nAbs. This observation is anticipated given that nnAbs typically target non-functional forms of viral surface proteins, the preponderance of which on infectious virions is likely to be less than functional forms. Such forms may be relatively abundant on infected cells, but then antiviral activity will only become effective once cells are indeed infected, thereby losing an advantage of nAbs to "strike early" in the infectious cycle. In any case, a focus on nAbs for passive administration is well justified by most literature, but nnAbs are also considered where significant below.

The application of nAbs in the clinic

Respiratory syncytial virus (RSV): many potential lessons

RSV is a respiratory virus with many potential lessons for SARS-CoV-2. The first and still the only antiviral nAb in widespread clinical use is the anti-RSV antibody palivizumab, marketed as Synagis, which is employed for the prevention of high-risk disease in high-risk infants [12,13]. First approved in 1998, palivizumab soon replaced RSV hyperimmune globulin (RespiGam) and its use was estimated to reduce hospitalizations in the US by about 55%. The antibody, directed to the surface F glycoprotein of RSV, was isolated by mouse immunization followed by humanization (CDR grafting) to generate a human IgG1 molecule. The humanized antibody bound F protein with an affinity of about 1 nM and was shown to neutralize laboratory-adapted strains from the two major RSV subtypes, A and B, in 3 different assays: a plaque assay, a microneutralization assay, and a fusion inhibition assay [14]. IC50s (Ab concentrations that reduce infectivity by 50%) for the two viruses were similar at ~2 μg/ml, ~0.1 μg/ml and ~0.2 μg/ml, respectively in the 3 assays. The antibody was shown to effectively neutralize a range of clinical isolates. Next, the antibody was evaluated in the cotton rat model of RSV infection and shown to offer essentially complete protection (a 99% reduction in lung viral titer) against intranasal challenge at an I.V. dose of 2.5mg/kg corresponding to a serum concentration of approximately 30 μg/ml at the time of challenge and a serum neutralizing titer of about 1:380. It was suggested that the plaque assay was less predictive of protection than the other two assays and indeed a serum concentration very roughly two orders of magnitude greater than the in vitro neutralization IC50 is associated with complete protection against a number of viruses in a number of animal models [15,16]. For palivizumab, it is recommended that antibody titers remain above about 30 μg/ml for protection from disease. Given the half-life of the antibody, this recommendation translates into a dose of 15mg/kg given monthly to at-risk infants throughout the RSV season, usually to a maximum of 5 doses. Importantly, although neutralization escape variants are readily generated in the lab, clinical failure of prophylaxis due to the emergence of palivizumab-resistant virus has not been reported in approximately 20 years of post-marketing analysis [17].

The requirement for multiple doses of palivizumab stimulated a search for an RSV nAb with greater potency and longer half-life that could be used ideally as a single dose to cover the RSV season. The initial candidate, motavizimab, was generated by in vitro affinity maturation of palivizumab to generate an

antibody with approximately 10-fold higher affinity for the F glycoprotein and 10-fold higher neutralization potency. Improving affinity against recombinant proteins that mimic viral surface proteins by in vitro approaches is now a much-sed approach to generate nAbs of higher potency. However, in this case, the improvement in neutralization potency was not translated into an improvement in protective efficacy in a clinical trial, where more adverse effects than with palivizumab were observed. The FDA voted against approval and work on the antibody was discontinued [18]. Another improved nAb, suptavumab, failed to meet efficacy end points based on reduced efficacy against an RSV B strain containing two amino acid substitutions in the Ab binding site [19]. Next, a potent nAb was generated by direct neutralization screening of supernates of immortalized B cells from an RSV-seropositive individual [20]. The antibody helped to define the pre-fusion conformation of F glycoprotein that led to the development of a very promising RSV vaccine [21]. Of note, the development of an RSV vaccine has a difficult history: an inactivated virus vaccine led to enhanced disease in vaccinees and was associated with the induction of nnAbs [22]. The potent antibody was further improved in terms of affinity for F glycoprotein and neutralization by engineering to yield MEDI-8897 (later nirsevimab), which was shown to give >2 logs improvement in neutralization titer and a nearly 1 log improvement in in vivo activity relative to palivizumab [23]. The antibody was also engineered with the LS mutations to extend half-life and entered Phase II trials in which it was shown to provide protection throughout an RSV season as a single injection of 50 mg [24]. Incidence of hospitalization was reduced by nearly 80%. Importantly, in the various studies of passively administered RSV nAbs, there has been no evidence of antibody-enhancing infection or disease. Anti-drug antibodies (ADA) responses have been low. In the recent trials of nirsevimab, 5.6% of participants who received antibody had ADA responses compared with 3.8% of placebo participants. Serum concentrations of nirsevimab were similar in participants who were positive and those were negative for ADA. No adverse effects in treated participants with ADA were associated.

In contrast to successful prophylaxis, palivizumab and motavizumab were not effective therapeutically in established RSV infection [13] [25]. This result agrees with dogma that suggest antibodies are generally relatively ineffective in established viral infection. However, as more and more highly potent antibodies ("super-antibodies") are investigated, this dogma is increasingly being challenged [2].

A respiratory virus such as RSV could, in principle, be treated by an aerosol-delivered nAb preparation provided the virus was anatomically accessible. It has been proposed that camelid-derived single-domain antibodies (sdAbs) could be useful as they contain a single heavy-chain variable domain. Because of their small size and high solubility and stability, sdAbs could be rapidly delivered the sites of respiratory virus infection via inhalation [26]. Prefusion F-protein-specific sdAbs that show up to 180,000 times greater neutralization potency than the first generation of RSV sdAbs have been identified and may offer even greater therapeutic benefit [27].

The demonstration of broadly neutralizing antibodies to highly antigenically variable viruses such as HIV and influenza virus, encouraged attempts to isolate nAbs to different members of a given virus family (pan-virus family antibodies). For respiratory disease, a nAb against RSV and metapneumovirus (MPV) was described that showed potent prophylactic activity against both viruses in an animal model [28]. Further nAbs with varying degrees of coverage to members of a given family of viruses have followed, including those to flaviviruses, filoviruses and coronaviruses [2].

In summary, RSV provides a system in which many potentially useful observations on the clinical use of nAbs have been made. The ability of an IM-administered nAb to protect against a respiratory virus in a very vulnerable human population was demonstrated. A well-chosen animal model predicted the human result. Improvements in neutralization in vitro were directly reflected in improvements in protective

ability in the clinic in one case, but not in two others. Highly potent nAbs have helped guide the design of a new generation of RSV vaccines (reverse vaccinology 2.0 [29,30]). Therapy by the prototype RSV nAb was less successful than protection. No problems with antibody-triggered enhanced disease or ADA have been encountered in the use of RSV nAbs to date. The prototype RSV nAb has been used for 20 years prophylactically without emergence of neutralization escape variants, but another nAb failed in the clinic because of neutralization resistance. RSV-specific small antibody constructs may be useful in therapy via aerosol delivery. The feasibility of nAbs able to act against multiple members of a family of viruses has emerged.

Human immunodeficiency virus (HIV): a highly antigenically variable virus and a pioneering field

The enormous antigenic diversity of HIV presents huge problems for nAb treatment and vaccine development and some of the solutions benefit virology generally. Clinically useful nAbs must be broadly neutralizing (bnAbs), i.e. able to neutralize a significant fraction of global isolates, and many studies have shown that there is sufficient conservation in exposed sites on the HIV Env trimer spike to permit broad neutralization. The first generation of bnAbs isolated in the 1990s was investigated in vivo and shown to protect at high concentrations in nonhuman primates (NHPs), but to have limited therapeutic value in small animal models. The second generation of much more potent bnAbs beginning in 2009 was achieved due to the ability to isolate Abs from rare B cells and from donors with favorable serum neutralizing Ab responses [2]. A notable feature of the second generation of bnAbs, also seen in other cases of nAbs aiming to neutralize a diversity of viruses, is a trade-off between potency and breadth [31]. The most potent bnAbs (IC50s in the range of 10-100pM) neutralized 60-80% of a panel of global isolates whereas the broadest antibodies that neutralized up to 99% of global isolates more often had IC50s of about 1nM. These second-generation bnAbs were able to protect NHPs at much lower concentrations than first generation bnAbs, mirroring the observations with RSV, of a tight correlation between neutralization and protection. Again, fully protective serum neutralizing antibody titers were 2-3 logs higher than in vitro neutralizing titers. A comprehensive examination of all the nAb NHP protection data [16] showed, in a logistic model that adjusts for bnAb epitopes and challenge viruses, that the serum ID50 (serum dilution that produces a 50% reduction in infectivity) had a highly significant effect on infection risk ($p < 0.001$). The estimated ID50s to achieve 50%, 75%, and 95% protection were 91, 219 and 685 respectively. Vaccine-induced nAb protection in NHPs was consistent with passive nAb studies and showed more than 90% protection at ID50s greater than approximately 1:500. It is worth noting a common caveat of animal model protection studies-the use of relatively high dose viral challenge to ensure that all control animals become infected and keeping the number of animals per study manageable. Certainly, the viral challenge dose typically used in NHPs is much higher than the average dose of HIV to which humans are believed to be exposed. The HIV field has approached this problem by development of a repeated low dose challenge model. Indeed, one study suggested that significantly lower serum nAb concentrations were required to offer notable protection than was required to offer sterilizing protection against high-dose viral challenge in NHPs [32].

Treatment of established infection in NHPs with more potent second-generation HIV bnAbs was more promising than treatment of humanized mice with first generation bnAbs [33]. With monotherapy, rebound occurred relatively quickly in the form of a virus identical to the challenge virus (no neutralization escape) for most animals. However, for animals with low viral loads, rebound was delayed for much longer periods. With bnAb cocktails, rebound could be further delayed giving sustained control of virus. It is suggested that this type of control is due to T cell immunity [34,35], either enhanced via better antigen presentation through immune complexes with bnAbs (a so-called “vaccinal effect”) or because the dampening of viral replication allows the T cell response to “catch up” with viral diversification.

Several potent HIV bnAbs are now in the clinic [36]. A large-scale protection study involving the bnAb VRC01, the Antibody Mediated Protection (AMP) study [37], and enrolling 2,700 volunteers in the US, South America and Europe and 1,900 volunteers in Africa will report in Fall 2020. The Ab was given at two doses, 10 or 30 mg/kg, every 8 weeks for a total of 10 IV infusions. Repeat dosing is designed to maintain serum levels of nAb at many multiples of the in vitro neutralization titers against the majority of a panel of HIV isolates. A feature of the trial is to study the nature of breakthrough viruses and serum levels of nAbs in treated volunteers to investigate whether "sieving" effects occur and potentially establish the serum levels of nAb that provide protection in humans.

Therapy in human trials with a cocktail of first-generation bnAbs was disappointing and showed little or no control of virus infection. Monotherapy using potent second-generation bnAbs showed transient viral control with the emergence of escape variants mirroring the results for small-molecule drugs. Combination therapy of two potent bnAbs was far more effective in viremic individuals but did not completely suppress viremia for an extended period [36]. If individuals were treated with anti-retroviral therapy (ART) and then an antibody combination, long-term suppression of virus could be achieved in a significant fraction of individuals. A novel way to combine bnAbs as single reagents, and bring down costs, is the generation of bispecific and now trispecific antibodies [38]. Another novel approach to passive immunization that may facilitate the use of mAb combinations is genetic i.e. Abs introduced as DNA, mRNA or viral vectors [39-44].

The role of antibody effector functions has been investigated in both protection and therapy in NHP models of HIV infection. A role for interaction of antibody with Fc receptors (FcR) in protection using a first [9] but not a second generation bnAb has been reported [6]. A role for FcR interaction in a therapeutic setting was suggested by two studies although the effects were not overwhelming and enhancing FcR interaction paradoxically led to a decreased FcR-dependent effect [5,7]. Of note, attempts to date to convincingly demonstrate protection against SHIV in the macaque model by passive transfer of nnAbs have failed [45].

Many of the HIV bnAbs have unusual features including high levels of somatic hypermutation (SHM), including in framework regions, and very long CDRH3 regions. There were initial concerns that these features would lead to enhanced ADA responses when the bnAbs were used clinically. These fears do not appear to have materialized.

Overall, because of its huge antigenic variability and the extremely extensive coverage of the HIV Env spike with glycans, HIV presents a severe problem for the development of effective nAbs for prophylaxis or therapy. Nevertheless, the ability to look through the Ab responses of many donors and mine the most promising to great depth has allowed the isolation of many potent bnAbs against several relatively conserved regions of the spike. In protection studies, bnAbs can provide complete sterilizing immunity in the NHP model. The combination of high potency and half-life extension has triggered plans to use bnAbs as prophylactic reagents given perhaps subcutaneously only once every 3-6 months. The therapeutic efficacy of bnAbs suggests, as combination formulations, they may find use to permit extended periods off ART and also perhaps as part of HIV cure strategies. The consideration of nAbs in these challenging roles in HIV infection illustrates how perceptions of the clinical scope of anti-viral nAbs have changed in the last decade. Given the prevalence of HIV infection in low and middle-income countries, attention has begun to focus on large-scale production of Abs at much lower cost [46]. Importantly, HIV nAbs are central to current approaches to rationally design an HIV vaccine.

Influenza virus: another highly antigenically variable virus

As for RSV, influenza virus is a respiratory virus with potential lessons for SARS-CoV-2 nAb treatment. In its high antigenic variation, influenza virus resembles HIV and there is a similar search for bnAbs and immunogens that would induce such bnAbs ("a universal flu vaccine"). However, there also major differences in the demands placed on bnAbs. It is likely that influenza virus bnAbs could be effective in prophylaxis by blunting infection, and sterilizing immunity would not be required as suggested for HIV. Moreover, some degree of protective activity may originate from cross-reactive nAbs elicited by earlier infection with different strains of influenza. In terms of therapy in acute infection, bnAbs would not face the huge viral diversity present in a single chronically HIV-infected individual and the risks of viral escape would be expected to be correspondingly much less. SARS-CoV-2 would be expected to be similar to influenza virus and distinct from HIV in this regard. Of course, over a very large population of infected individuals, escape by any virus under nAb monotherapy pressure is possible. Finally, for influenza virus, there is always the concern that a pandemic will result from a novel reassortment of a human virus with the large reservoir of zoonotic viruses, particularly in aquatic birds and pigs. There is then a case for stockpiling influenza bnAbs that would have some anti-viral activity against a newly emerging strain and offer immediate prophylactic and therapeutic tools in the event of a pandemic. The same case can be made for stockpiling bnAbs or pan-virus family Abs for a range of viruses, including coronaviruses.

Two predominant classes of bnAbs to influenza have been identified that illustrate the trade-off that often arises between antibody neutralization potency and breadth [47]. BnAbs to the globular head of the spike hemagglutinin (HA) protein are potent, neutralizing replication-competent and pseudoviruses at low concentrations (5-500 ng/ml). However, the breadth of "head" bnAbs is often limited and escape generally occurs, at least in vitro, relatively easily. BnAbs to the stem neutralize pseudoviruses very well but are much (100-1000-fold) less effective against replication-competent viruses, consistent with the tighter packing of HA molecules in the latter case that restricts access to the stem region. On the other hand, stem bnAbs are broader-antibodies that neutralize both group 1 and group 2 influenza A viruses have been isolated-and escape from these Abs in vitro is limited. Of note, stem but not head bnAbs appear to depend on FcR-mediated effector functions for protective activity in a mouse model [48]. A phase IIB study of the use of an anti-stem Ab, CT-P27, in acute influenza A infection has been carried out [49]. The Ab, given at either 90 mg/kg or 45 mg/kg on day 1, reduced the time to resolution of symptoms and fever from 5.7 days in the placebo group to 3.7 days in both Ab-treated groups. A phase II study of the anti-stem Ab, CR6261, given at 50 mg/kg on day 1 after challenge with influenza H1N1 was not superior to placebo in any of a series of endpoints, except one; the proportion of participants who experienced influenza symptoms was reduced from 93% to 75%[50]. Therefore overall, the clinical effects of passive anti-stem Abs to date have been very moderate.

As with HIV, novel strategies have been developed to present multiple antigenic specificities in the context of single molecules. A multi-domain construct incorporating two influenza A and two influenza B sdAbs was generated and shown to have potent and broad neutralization and offer protection in mice against both A and B viruses [51]. These multidomain antibodies targeted both the head and the stem.

Non-neutralizing Abs have shown some interesting anti-influenza virus activities in vitro and in vivo. Abs to a trimer interface in the HA head region that appears occluded on the native HA spike do not neutralize virus but do bind to infected cells and do show protection in mice [48,52 53]. Similarly, Abs to neuraminidase (NA) and the matrix protein (M2) that are present on influenza virions at lower (NA) to much lower (M2) density than HA are non-neutralizing but are protective in mouse studies [47]. An antibody to M2 was given to volunteers I day after challenge with influenza A when some benefit was argued in terms of milder

symptoms (35% reduction in a quantitative assessment) [54]. Antibodies have been described to NA that are not neutralizing in the classic sense of blocking viral entry but do block viral egress from target cells in a modified in vitro assay and do protect mice [55].

Overall, influenza virus studies describe highly strain cross-neutralizing Abs that could find both prophylactic and therapeutic application and the feasibility, in principle, of a universal flu vaccine. Further, since humans are the only host for influenza B, it may be possible to eradicate this virus

Ebola virus: an acute, extremely rapidly replicating and deadly virus

Ebola virus infection provides a different sort of challenge to nAbs than the highly antigenically variable viruses with potential lessons for SARS-CoV-2. The virus is highly infectious and highly lethal e.g. exposure to a few pfu of virus is said to be fatal for a monkey in about 10 days [56]. Viral replication is explosive producing very high titers systemically and multiple organ failure. Although the virus is typically uniformly fatal for monkeys, the fatality rate in humans varies widely between about 25 and 90%, depending upon the viral species, strain and outbreak conditions

The first nAb was isolated from a sample from a survivor of the Kikwit outbreak in 1976 [57]. The antibody protected against challenge in a guinea pig model, but did not protect in monkeys [58]. In retrospect, the reason may have been less than complete neutralization of virus because of some heterogeneity in the epitope recognized [56].Incomplete neutralization is a feature of a number of nAbs to SARS-CoV-2, especially non-RBD S-protein nAbs, and should be interpreted as a potential red flag for clinical applications. Later, several mAbs to Ebola virus were isolated and shown to be effective in monkeys when given several days after infection and after the onset of symptoms [59-62]. A large-scale comparative Ab study postulated neutralization and effector function as the best correlates of infection in a mix of animal models [63]. Of note with regard to neutralization of Ebola virus is that the virus enters the cytoplasm by a series of steps that can complicate understanding of the stage at which Abs is acting anti-virally. Macropinocytosis of virions into lysosomes is followed by removal of the glycan cap and mucin-like domain of the surface glycoprotein by host cathepsins, exposure of the receptor binding site, interaction with receptor and then fusion with the endosomal membrane.

Two Ab preparations were recently investigated in humans for therapeutic activity in an Ebola virus outbreak in West Africa (the PALM trial). The first preparation was a cocktail of 3 Abs; one potent nAb (IC50 ~0.2 nM) in pseudovirus and replicating virus assays, one nAb that showed incomplete neutralization in the pseudovirus assay and no neutralization in the replicating assay and one that showed no neutralization in either assay [60]. The cocktail was protective in monkeys even when given after symptom onset. The second Ab preparation is a single potent nAb (IC50 ~ 0.6 nM) that again was shown to protect monkeys even when given as late as 5 days after infection [59]. The cocktail was given as a single IV dose at 150mg/kg and the single Ab as a single IV dose at 50 mg/kg. In a randomized controlled human trial, the number of deaths per number of infected patients (CFR) was about 50% for optimized supportive patient care (oSOC) together with another Ab mix used in a previous outbreak or used with remdesivir but 34% for the single Ab and 35% for the 3-Ab cocktail. When patients arrived early for treatment, the CFR was reduced to about 10%, suggesting an optimal window for treatment, either because of limits in the mAbs used or because late stage disease is refractory to treatment. The results were seen as a considerable success, especially given that many infected individuals did not seek medical help until 11-12 days after likely exposure to virus, often deep into the illness with multi-organ failure. It is nevertheless argued that there is still great room for improvement in terms of Ab treatment of Ebola virus infection [64].

Finally, Ebola virus occurs as several distinct subtypes and the above mAbs are specific to EBOV. Therefore, a pan-ebolavirus reagent was sought and generated as a cocktail of two bnAbs that were able to resolve infection with either EBOV, Sudan (SUDV) or Bundibugyo (BDBV) in both ferrets and monkeys [61].

Overall, the over-riding lesson from the Ebola virus field is that mAb therapy can influence the course of infection of an explosively replicating, systemically spreading virus.

Coronaviruses: what are the key lessons from other viruses?

First, it is worth emphasizing the point made initially that no two nAbs or viruses behave identically and therefore all extrapolations from other studies and other fields bear this caveat. This is expected given that antibodies and viruses are both the products of mutation and selection and are hugely diverse and continually evolving. Nevertheless, certain guiding principles can be proposed for the development of nAbs for prophylaxis and therapy of SARS-CoV-2 and for a nAb-based COVID-19 vaccine.

1. *NAb potency is a key attribute as illustrated above*. It can be considered that we are now entering the fourth age of passive Ab immunization in infectious disease. The first age was immune sera at the beginning of the 20th Century, the second was purified immunoglobulin in the 1940s, the third was monoclonal antibodies beginning in the 1990s and the fourth is now monoclonal antibodies of remarkable potency and breadth ("super-antibodies"). These super-antibodies typically neutralize viruses in the pM range (ng/ml) and should be the target for nAbs to SARS-CoV-2. Promisingly, several nAbs isolated from SARS-CoV-2-infected donors show this level of potency. Studies on a number of viruses indicate that protection in vivo is typically associated with serum nAb concentrations 2-3 logs higher than in vitro neutralization titers, suggesting a serum target of µg/ml for SARS-CoV-2 nAbs. A cautionary note from studies on other viruses is that neutralization may not always guarantee protective or therapeutic activity for reasons that, at this stage, are poorly understood. Another cautionary note is that incomplete neutralization in vitro, i.e. where a fraction of a virus population is not neutralized by the nAb, is an indicator that the nAb may not be suitable for clinical use.
2. *NAb breadth is potentially important*. Abs that can neutralize many viral variants by recognizing relatively conserved regions on viral spikes may have advantages in both protection and therapy. Broad nAbs are more likely to be effective against the diversity of circulating isolates and less likely to select for neutralization escape variants during therapy. However, there may be a trade-off between breadth and potency. Indeed, for SARS-CoV-2 nAbs, the most potent nAbs appear directed to the ACE2 binding site (the RBD-A site), but they are not cross-neutralizing for SARS-CoV-1. SARS CoV-1/2 cross-neutralizing antibodies directed to an adjacent RBD-B site are typically less potent. Nevertheless, HIV studies have underlined the potential to isolate nAbs with outstanding breadth and potency given extremely intensive searches and adoption of in vitro affinity maturation approaches. Breadth and neutralization resistance can be enhanced by the use of Ab cocktails and/or bi- or tri-specific Abs.
3. *Antibody effector function can be crucial*. There are many clear instances where antibody effector function is required for nAb anti-viral activity in vivo. If there are clearly demonstrated concerns with ADE, it may be appropriate to eliminate FcR binding, but it should be borne in mind that this could be highly detrimental for nAb protective and therapeutic activities in vivo.
4. *Antibody-dependent enhancement (ADE) of disease has only been demonstrated for flaviviruses*. Data for other viruses in vivo is lacking.
5. *Anti-drug antibodies (ADA)*. Repeated use of nAbs as a prophylactic may create ADA, although it should be noted that most potent SARS-CoV-2 nAbs isolated to date have minimal somatic

hypermutation. The AMP trial will report soon using the highly mutated VRC01 HIV bnAb given repeatedly at relatively high dose.

6. *Novel antibody fragments may have a role against respiratory pathogens*. Single domain antibodies (sdAbs) have shown promise against RSV and could be deployed against SARS-CoV-2.
7. *Broad virus family nAbs can be generated*. Pan-sarbecovirus nAbs could be used not only against existing SARS coronaviruses but might provide some level of efficacy against coronaviruses that may emerge in the future.
8. *NAbs are guiding vaccine design.* The design of a new highly promising RSV vaccine began following the discovery of a nAb to a new site (site Ø) on the prefusion form of the F glycoprotein. HIV vaccine development is focused on the design of immunogens to target sites defined by bnAbs. For SARS-CoV-2, nAbs have helped define the importance of the RBD and suggest templates for the design of immunogens to induce the most potent nAbs and those with the greatest cross-reactivity with other coronaviruses.

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Captions to Table and Figure

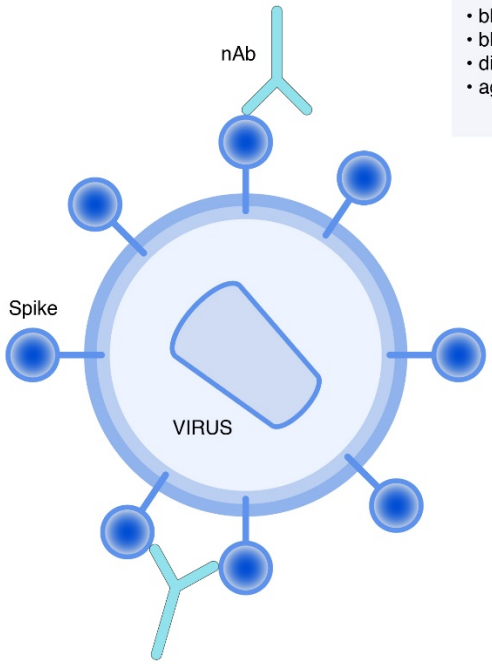
Table 1: A selection of mAbs in clinical trials for the treatment or prophylaxis of viral infections. Much more extensive information is available at https://www.antibodysociety.org/ and at https://wellcome.ac.uk/sites/default/files/expanding-access-to-monoclonal-antibody-based-products-appendix.pdf.

Antibody	Virus	Antibody isolation technology	Target	Stage of development	Trial Sponsor	Indication
ZMapp	Ebola virus	3 mAb cocktail; chimerized mouse Abs	Viral Env glycoprotein	Phase II and III	Mapp Biopharmaceutical	Treatment of Ebola virus infection after exposure
MAb114	Ebola virus	Human B cell isolation	Viral Env glycoprotein	Phase II and III	NIAID	Treatment of Ebola virus infection after exposure
REGN-EB3	Ebola virus	3mAb cocktail; humanized mice	Viral Env glycoprotein	Phase II and III	Regeneron	Treatment of Ebola virus infection after exposure
PRO 140	HIV	Immunization and humanization	CCR5	Phase III	Cytodyn	Treatment of HIV-1 infection
Ibalizumab	HIV	Immunization and humanization	CD4	Approved	TaiMed Biologics	Treatment of HIV-1 infection
UB 421	HIV	Immunization and humanization	CD4	Phase II	United Biomedical	Treatment of HIV-1 infection
VRC01	HIV	Human B cell isolation	HIV Env	Phase IIB	NIAID	Prevention of HIV-1 infection

N6-LS	HIV	Human B cell isolation	HIV Env	Phase I	NIAID, GSK	Prevention of HIV-1 infection
10-1074-LS-J1 and 3BNC117-LS-J1	HIV	Human B cell isolation	HIV Env	Phase I/II	Rockefeller University	Prevention and treatment of HIV-1 infection
ePGDM1400 and ePGT121	HIV	Human B cell isolation and affinity maturation	HIV Env	Preclinical	IAVI	Prevention of HIV-1 infection
10E8.4/iMab	HIV	Human B cell isolation, bispecific	HIV Env	Phase I	ADARC	Treatment and prevention of HIV-1 infection
VIS 410	Influenza virus	Unknown	Influenza virus HA	Phase II	Visterra	Treatment and prevention of influenza A virus infection
MHAA 4549A	Influenza virus	Human B cell isolation	Influenza virus HA	Phase II	Genentech	Treatment of influenza A virus infection
CT P27	Influenza virus	Human B cell isolation	Influenza virus HA	Phase II	Celltrion	Treatment and prevention of influenza A virus infection
CR6261	Influenza virus	Phage display	Influenza virus HA	Phase II	Crucell; J&J	Treatment and prevention of influenza A virus infection
CR8020	Influenza virus	Human B cell isolation	Influenza virus HA	Phase II	Crucell	Treatment and prevention of influenza A virus infection
RG 6024	Influenza virus	Human B cell isolation	Influenza virus HA	Phase I	Genentech	Treatment of influenza B virus infection
MEDI 8852	Influenza virus	Human B cell isolation	Influenza virus HA	Phase II	MedImmune	Treatment of influenza A virus infection
TCN 032	Influenza virus	Human B cell isolation	Influenza virus M2e protein	Phase II	Theraclone Sciences; Zenyaku Kogyo	Treatment of influenza A virus infection
m102.4	Nipah and Hendra virus	Phage display	Viral Env glycoprotein G	Phase I	Uniformed Services University and Queensland Dept of Health	Prevention and treatment of Nipah and Hendra virus infections
Twinrab	Rabies virus	2 mouse mAb cocktail; immunization	Viral Env G protein	Approved in India	WHO; Zydus Cadila	Prophylaxis after exposure to rabies
Rabishield	Rabies virus	Humanized mice	Viral Env G protein	Approved in India	Serum Institute of India; MassBiologics	Prophylaxis after exposure to rabies
Palivizumab	RSV	Immunization and humanization	Viral fusion protein	Approved	MedImmune	Prophylaxis in high-risk infants
Nirsevimab	RSV	Human B cell isolation and in vitro maturation	Viral fusion protein	Phase IIB	Sanofi, Astra Zeneca	Prophylaxis in all infants

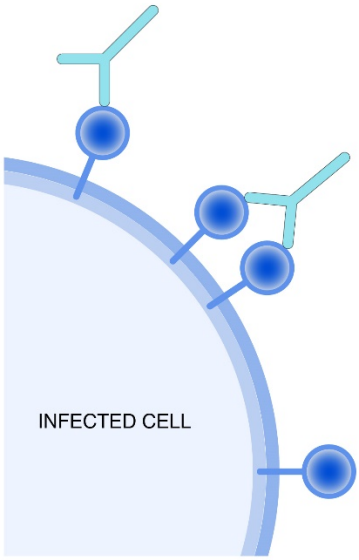
Figure 1: Schematic of activities of nAbs against free virus and virally infected cells.

a) nAb activities against free virus



in vitro (neutralization)	in vivo
• block viral attachment • block fusion • disassemble spike • aggregate virions	• in vitro mechanisms • C^-: enhance blocking, virolysis • phagocytosis • virus sequestration

b) nAb activities against virally infected cells



in vitro	in vivo
• ADCC • ADCP • virus egress	• in vitro mechanisms

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SESSION 5

Assay standardization

Presenter
Erica Saphire, Ph.D., La Jolla Institute for Immunology

Moderator
John Dye, Ph.D., USAMRIID

Panelists
Holly Soares (Pfizer)
Bette Korber (Los Alamos National Laboratory)
Steven Kozlowski (CDER/FDA)
Hendrik Neubert (Pfizer)

Topic presentation and discussion: Assay development- Assay Standardization

John Dye, Ph.D., USAMRIID

Purpose: Bring together scientists, clinicians and regulatory officials to discuss the risks, scientific gaps and neutralizing antibody countermeasures to SARS-CoV-2 infections. Specifically, this session focused on the *in vitro* screening characteristics that may predict efficacy and safety and the process for screening of nAb candidates and indicators of potential optimal combinatorial products.

Summary: In the presentation given by Dr. Saphire, it was clearly articulated the value added to all preparedness efforts for medical countermeasures in having standardized assays for assessment of reactivity, safety and efficacy. Doing so in normal scientific environment can be challenging, doing so in a pandemic environment can be even more challenging and even daunting. The presentation highlighted historical analysis that has been done assessing antibodies to other infectious diseases in standardized assays. Neutralization assays for HIV, RSV and EBOV, expectedly showed that the target cell and virus strain or isolate used in the assay were important and introduced some variability in the performance of these tests. This highlights the need for standardization of these variables in hopes of providing a direct comparison of products between laboratories.

The most robust comparison of multiple assays to date was performed by the Viral Hemorrhagic Fever Immunotherapeutic Consortium (VIC). Data gathered by this large team indicated that neutralization assay from multiple sources (live virus or pseudovirus) showed the greatest correlation with protection in animal models. Also, importantly highlighted was the fraction of un-neutralized virus remaining in these assays inversely correlating with the potency of these products *in vivo*. Following the successes of VIC, a new international collaboration has been formed called the Coronavirus Immunotherapeutic Consortium (CoVIC). The same parameters as were assessed by the VIC for Ebolavirus will be translated to novel SARS-CoV-2 antibodies by the CoVIC. This includes but is not limited to: binding, neutralization, structural biology, escape and surveillance, FC profiling (both serology and cellular studies), and *in vivo* modeling. Products can be provided to the CoVIC where they will be "blinded" and provided to multiple laboratories to complete the anonymized analysis. These anonymous results will be made publicly available in a CoVIC database. Discussion ensued following the presentation by the panelists that was focused in five specific areas:

1) The need to balance standardization of assays with openness and maintaining a rapid response in the face of a pandemic. Multiple panelists pointed out that these are not mutually exclusive but requires an openness of those involved to submit their candidates to an organization such as the CoVIC. The discussion of making a reference standard that could be used in comparing results or bridging results across laboratories was brought up several times. It was agreed that this would be an excellent resource for the present and future. The organizers stressed the importance of the OWS entities to consider depositing their candidate antibodies into the CoVIC and doing so expeditiously.
2) The discussion of intelligent design of antibody cocktails. The comment was made that the majority of the antibodies being assessed are very similar in binding domains and nature. Perhaps the analysis by the CoVIC will allow different combinations of synergistically acting antibodies to be coupled in experimental design. The pairing of non-redundantly binding or functioning antibodies needs exploration and discussion.
3) The field needs to learn from all the ongoing clinical trials and those slated to start in the future. Communication of results from clinical studies, early and often, may allow future studies to avoid the "pitfalls" and take advantage of the successes identified in these early studies. The studies highlighted were the convalescent plasmas studies, as well as the antibody studies that are initiated or soon to begin. The introduction of the vaccination of the population into the equation

was also discussed. Specifically, to monitor closely the immunization status of those individuals who may have received antibody treatment with subsequent vaccination for protection against SARS-CoV-2.

4) A better understanding of particular FC functionality that may be optimal for antiviral activity of anti-SARS-CoV-2 antibodies is needed. Thanks to efforts like the VIC and other researchers, we do understand some of the desired profiles of activity for other viruses, however bridging those findings to SARS-CoV-2 will be important. This understanding can lead to intelligent design of cocktails or manipulation of monoclonal antibodies to increase functionality such as antiviral activity.
5) A discussion on the D614G variant of SARS-CoV-2 and the potential need to assess countermeasures against this virus as it is now globally dominant was discussed. A better understanding of the different variant viruses in human population is needed in order to determine the importance of assessment against one particular variant vs. multiple variants.

In summation, while great strides have been made in understanding the importance of neutralizing antibodies specific for infectious diseases in general, the future effort by our scientific community will be generating the information specific to SARS-CoV-2. The availability of standardized assays or sites where standardized assays are available will be extremely important in moving forward desirable products.

Assay Standardization for Neutralizing Antibody
Why Different Labs Can Get Different Results and A Path Forward

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SARS-CoV-2, the novel coronavirus that causes COVID-19, has upended lives and livelihoods around the globe, with nearly 20 million infections, some with long-term consequences, and over 700,000 deaths in just seven months. Disruption in employment, education, travel and even simple gatherings has been unprecedented. Also unprecedented is the pace of progress in development of possible treatments for COVID-19. Multiple therapeutics including monoclonal antibodies (mAbs), small molecules and antivirals as well as vaccine candidates are currently in clinical trials. To support the development of these treatments, robust and reliable assays of therapeutic activity–at different levels of biocontainment–are urgently needed.

However, it is not always clear which laboratory assays of activity *in vitro* will best forecast human protection *in vivo*. For monoclonal antibody treatments, viral "neutralization", the ability of an antibody to block viral activity, particularly viral entry, in cell culture is thought to be a key indicator of success. Therapeutic antibody candidates have all been evaluated for neutralization. The key challenge, however, in comparing therapeutic candidates from different organizations, and across published studies, is that neutralization assays and assay results can vary widely. Even when a standard protocol is prescribed, inter-assay, inter-laboratory, or inter-operator variables can make it difficult to compare therapies or to draw absolute conclusions.

Different labs get different results.

Some differences among neutralization assays are clear: labs may use different viral strains, which grow to different titer or bear different sequences of the viral surface glycoprotein or spike that can affect the capacity of a given antibody to bind to the surface glycoprotein and inactivate entry. Other differences are associated with surface glycoprotein density or spacing, glycosylation patterns, producer or target cells, virus or cell passage number, and use of single-clone vs. uncloned viral stocks. Use of standardized assays can facilitate apples-to-apples comparisons among antibody candidates. To better understand which assay formats might best predict in vivo success, a number of groups have evaluated the range of possible neutralization assays, considering virus type, assay readout and inter-laboratory and intra-laboratory variables. However, when it is unclear which neutralization assay format best forecasts *in vivo* protection, analysis of neutralization using multiple approaches could be a more reliable indicator of efficacy.

We were asked by the Therapeutics Accelerator to build the international Coronavirus Immunotherapeutic Consortium (CoVIC) to understand and evaluate therapeutic antibodies against SARS-CoV-2. Key to this consortium are measures of neutralization and other antibody activities for samples run-side-by-side, in standardized assay formats. Here we put this study on SARS-CoV-2 in the context of prior efforts to evaluate and standardize neutralization assays for HIV-1, Ebola virus, RSV and current efforts of the CoVIC against SARS-CoV-2.

HUMAN IMMUNODEFICIENCY VIRUS (HIV-1)

Several groups have characterized HIV-1 neutralization assays, including the Laboratory Standardization Subcommittee for the Global HIV AIDS Vaccine Enterprise (GHAVE)[1], NeutNet[2,3], and others[4], each using a different strategy to compare different variables. GHAVE compared neutralization of primary HIV-1 isolates infecting PBMCs to that of pseudoviruses derived from corresponding HIV-1 isolates infecting the TZM-bl reporter line. This group found that the degree of correlation between authentic virus and pseudovirus assays was dependent on the reagents used for neutralization[1].

NeutNet, involving 18 different laboratories, compared multiple neutralization assay formats, each using the same neutralizing reagents [TriMab cocktail, mAb 447-52D, mAb 4E10, and sCD4 (soluble receptor)], and the same panel of viruses [11 different viruses representing a range of genetic subtypes and phenotypes] each provided from a common source. Some assays in NeutNet used uncloned, authentic HIV-1, while other assays used pseudotyped viruses. Quantification was based on detection of intracellular or extracellular p24, RNA level quantification or beta-galactosidase reporter gene expression. Although pseudovirus was in general more easily neutralized than authentic virus in these assays, the results varied significantly depending on both the virus and the neutralizing reagent used. Variables among HIV-1 neutralization assay formats that affected results include envelope spike density, glycosylation differences arising from cell types used for production, readout method or incubation time, with longer incubation of virus with antibody leading to increased neutralization activity[2,3].

Features of the target cell used also affected neutralization sensitivity as evidenced by the greater inhibition seen for macrophages as target cells relative to PBMCs. PBMC neutralization sensitivity varied based on whether the cells were from individual donors or were pooled from several donors. Neutralization sensitivity was also affected by the relative levels of chemokine receptors on reporter cells. Some antibodies required prior interaction of the glycoprotein with receptor or co-receptor to open the antibody epitope. These antibodies would appear less or more potent, depending on the cell line used and the ratio of cellular molecules. The ratio of chemokine/CD4 and other receptors involved in virus entry differs among target cells, and within an individual cell line or passage. In these studies, TZM-bl cells showed batch-to-batch variations in chemokine ratios and other receptors. Cell lines also differ in mechanism of virus uptake: TZM-bl reporter cells are more likely to take up HIV by endocytosis compared to PBMCs.

Features of the virus used can affect apparent neutralization performance. For example, all neutralizing reagents (antibodies and sCD4) analyzed exhibited poor IC_{50} (> 25 μg/ml) against QH0692 HIV-1 in 3T3 cells, whereas in HeLa cells, the IC_{50} improved substantially (4-13 μg/ml). In contrast, some antibodies neutralized 92UG024 HIV-1 similarly in both 3T3 and HeLa cells[2]. Virus type (e.g., single clone vs. uncloned) can also contribute to differences in neutralization due the presence of sequence variants. Neutralization of identical plasma stocks yielded a mean IC_{50} of 45 μg/ml against a pool of amplified clones obtained from a viral supernatant, whereas for single clones the mean IC_{50} was 1,284 μg/ml[3]. Given the high mutation rate of HIV-1, a pool of amplified clones rather than a single clone might better represent the quasi-species that could be present in a patient.

Another key variable is the ratio of infectious to non-infectious particles in a virus stock. All viral supernatants harvested from producer cells contain some percentage of noninfectious virions, which can be fully intact, but nonetheless lack functions needed for productive infection[5,6]. Noninfectious virions can arise during virus production due to mutations, mis-incorporation of viral components during assembly or budding, or the relative abundance of key host proteins in specific cell lines. Some deficits are manifested only during *de novo* viral production, so that interactions of the defective virion with neutralizing antibodies, attachment receptors, or entry/fusion receptors can compete with or disrupt interactions with infectious virus. In other cases, heterogeneity in glycosylation, trimer assembly, and/or surface distribution of viral glycoproteins can directly affect interaction with attachment receptors, entry/fusion receptors and neutralizing antibodies. The ratio of noninfectious or inert virus particles per infectious unit may vary among producer cells and transfection or inoculation methods. Furthermore, the ratio can differ between laboratories, and even between transfections performed within the same laboratory using the same protocol with the same producer cells. This ratio of noninfectious to infectious units ranges widely both within and among different virus species [5]. For adenovirus the range may be 20-100 noninfectious virions per infectious unit[7], whereas dengue virus can range from 3×10^3 to 7×10^4. Meanwhile, the alphavirus Semliki Forest virus has low ratios and narrow ranges of 1–2[7,8]. HIV-1 has a broad range of reported noninfectious to infectious particle ratios: 1–10^2 [9]; 10^2–10^4 [10]; 10^3–10^4 [11 12]; 10^2–10^4 [13]; ~10^5 [14]; and 10^4–10^7 [15], which can confound the interpretation of neutralizing assays across laboratories. The short infectivity half-lives at physiological temperatures and varied responses to thawing

and storage by HIV-1 and other retroviruses adds to the challenge of obtaining an even playing field to assess the efficacy of neutralizing antibodies [15,16].

Thus, meaningful comparison of the efficacy of neutralizing or therapeutic antibodies across laboratories or in the context of a global consortium requires normalization of viral challenge, not just through traditional titering of infectivity vs. volume, or by viral particle number or activity as is the case for p24 capsid (CA) or reverse transcriptase (RT) assays for HIV-1, but through understanding and normalizing the ratio of noninfectious to infectious virus used in the assay. Indeed, Todd et al. [4] observed that lab-to-lab variability among laboratories using the same neutralization assay protocol and the same viral sequence could be minimized when the same pool of pseudovirus stocks was distributed across the labs.

Across all the HIV-1 studies, the groups found that no single assay was capable of detecting the entire spectrum of antibody neutralizing activities. Further, it is unclear which *in vitro* assay best correlates with *in vivo* protection. Thus, the results of these studies indicate that a panel of neutralization assays can best capture the range of antibody activities needed for comprehensive evaluation of vaccine efficacy [2,3,17].

RESPIRATORY SYNCYTIAL VIRUS

The development of the neutralizing monoclonal antibody palivizumab against respiratory syncytial virus (RSV) provides an example of considerations for antibody-based therapeutics against a respiratory virus[18]. The RSV fusion protein (F) drives virus and host membrane fusion in RSV infection and is a prime antibody target. First-generation neutralizing antibodies targeting epitopes on F that existed on both the pre- and post-fusion conformations were replaced by more potent antibodies that bind only the prefusion form. Serum from mice and rhesus macaques immunized with a prefusion stabilized form of F contained antibodies that had substantially higher neutralizing antibodies compared to those isolated from animals immunized with postfusion F [19]. This result is similar to that seen for Lassa virus glycoprotein, in which neutralizing antibodies, which were thought to be rare, bind with preference to a stabilized, prefusion, trimeric form of the surface glycoprotein [20].

RSV also serves as an example for the importance of inclusion of reference standards in neutralization assays. A collaborative study to examine inter-laboratory variability in titers of neutralizing antibodies against RSV subtypes A and B showed that agreement in results obtained by participating laboratories was markedly improved when the results were normalized relative to those for reference standards [21,22]. In addition, RSV microneutralization assay outcomes are affected by the substrate cells used and the presence or absence of complement [23]. In particular, the human lung carcinoma cell line A549 detected higher neutralizing antibody titers and was less affected by the presence of complement than were Vero or HEp-2 cells that are frequently used as substrate cells in neutralization assays.

EBOLA VIRUS

Studies to compare neutralization assays and other assays of antibody performance for Ebola virus were undertaken in response to a puzzling set of observations in the research field. In 2013, the then most potent known neutralizing antibody failed to protect non-human primates from Ebola virus challenge, while a cocktail of three poorly or non-neutralizing antibodies protected NHPs from challenge. A group of investigators formed the Viral Hemorrhagic Fever Immunotherapeutic Consortium (VIC) to determine what information was missing: Were neutralization assays not performed in the most predictive way? Did neutralization *in vitro* not correlate with protection *in vivo*? What other features beyond neutralization did correlate with protection? Was the greater protection related to the delivery of a cocktail rather than a monotherapy?

The VIC study, supported by a Center of Excellence in Translational Research (CETR) grant from the National Institute of Allergy and Immunology (NIAID), included academic, industry and government laboratories across five continents in a large, multidisciplinary study of a panel of 168 mAbs against Ebola virus (EBOV) glycoprotein (GP) raised in different laboratories and selected in those laboratories using different criteria[24]. The aim was to evaluate both the field's antibody candidates and antibody assays at the same time to determine which antibody candidates offered the best protection and which *in vitro* assays best predicted *in vivo* success. Antibodies donated by participating laboratories were anonymized through the assignment of code names, shipped to participating labs, and then examined side-by-side in a battery of standardized assays to determine which of 32 different antibody features correlated with *in vivo* protection beginning with a mouse model of EBOV infection. The VIC conducted biochemical and structural biology analyses, and also profiled glycan patterns, risk of escape mutations, and complementary contributions to protection from the Fc[25–27].

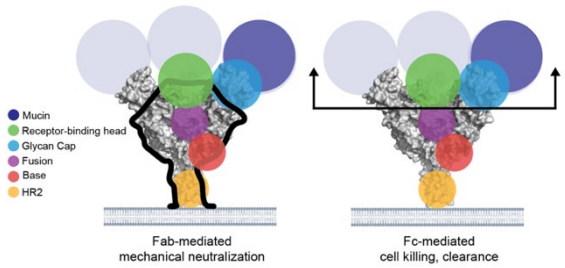


Figure 1. Fab- and Fc-mediated activities associated with Ebola virus GP epitopes. The black line outlines the region of GP that remains after cleavage (left). Antibodies targeting epitopes that are retained after cleavage are associated with mechanical neutralization. Epitopes in the upper and outermost regions (right, above the line) are associated with Fc-mediated functions.

The VIC compared three independent neutralization assays: i) rVSV-EBOV, a recombinant vesicular stomatitis virus pseudotyped to display EBOV GP on the virus surface that can be performed at BSL-2 containment; ii) ΔVP30 EBOV, a replication-incompetent EBOV in which the VP30 protein needed for replication is supplied *in trans*[28], performed at BSL-2+/3 containment; and iii) authentic EBOV in a microneutralization format performed at BSL-4 containment. The VIC also used the rVSV system to measure the fraction of viral particles left un-neutralized at the highest antibody concentration tested. Complementary Fc-mediated activities of the mAbs were surveyed using high-throughput systems serology to profile: i) phagocytic activity (ability of mouse and human monocytes and neutrophils to phagocytose particles bearing GP trimers incubated with a given mAb; four readouts); and ii) activation of human natural killer (NK) cells (three readouts: cell-surface expression of the lysosome marker CD107 and secretion of the inflammatory chemokine MIP-1β and cytokine IFN-γ).

Results of these parallel assays revealed several clear trends. First, epitopes that were likely to exhibit neutralization in cell culture tended to cluster in the receptor-binding core of the GP that remains after processing and governs viral entry (Figure 1, left, area within the black line). Second, epitopes that had the highest phagocytic functions localized to the uppermost regions–those farthest from the base of the GP near the virus membrane–that included the head region that include the receptor binding domain as well as the glycan cap and mucin-like domain that are cleaved away during endosomal processing (Figure 1, right). Meanwhile, antibodies that could activate NK cells had epitopes that were distributed across the GP.

Neutralization, potent antibodies and the most predictive assays:

Of the168 mAbs in the VIC panel, 49 were highly protective in a mouse model of EBOV infection. Of these, 20 exhibited neutralization in all three platforms, and indeed, the most potent neutralizers ranked highly in every neutralization assay format. Those antibodies that had more moderate neutralization capacity often neutralized in some formats but not all. The most potent antibodies consistently neutralized, and were likely protective because they neutralized[25].

Overall, outcomes for neutralization activity were the strongest univariate predictors of in vivo protection. Among the neutralization assays compared, the ability to neutralize authentic virus in the BSL-4 assay (Dye lab, USAMRIID) best predicted in vivo protection (Figure 2). For this microneutralization assay, Vero E6 cells were seeded in 96-well plates and incubated with a mixture of 50 µg/mL antibody and authentic EBOV at a MOI 0.2 [29]. The cells were fixed and the percentage of infected cells was determined using an indirect immunofluorescence assay with EBOV-specific human mAb KZ52 and anti-human IG conjugated to Alexa Fluor 488 as the primary and secondary antibody, respectively. The percentage of inhibition for each antibody was determined by comparing fluorescence in the presence of antibody to that of control cells incubated with only media.

The second-most predictive feature was the fraction of virus left un-neutralized by rVSV at the highest concentration antibody tested, 50 µg/mL (the un-neutralized fraction was only evaluated in the rVSV assay and not in the BSL-4 or BSL-2+ assays). Antibodies that left some percentage of virus un-neutralized typically failed to achieve high levels of in vivo protection. These antibodies tended to recognize epitopes that were variable in conformation or glycosylation state. As one example, if a loop or an N-terminus of the glycoprotein could occupy an "up" position that allows antibody binding as well as a "down" position that disallows antibody binding, incomplete occupancy and incomplete neutralization could result. For an epitope containing a potential N-linked glycosylation sequon, a glycan may be attached in only part of the glycoprotein population. If the glycan is required for binding or to block binding, the antibody may or may not be able to bind those copies of GP and in turn affect neutralization activity.

The ability of rVSV itself to forecast neutralization ranked below that of BSL-4 EBOV, fraction unneutralized and the BSL-2/3 replication-deficient ΔVP30 assay. The moderate predictive value of rVSV was unexpected given that this assay was performed using a nine-point concentration curve (50 µg/mL and eight dilutions), while the BSL-4 and BSL-2/3 assays were performed using a single antibody concentration, the maximal 50 µg/mL. All three assays used Vero cells (Vero for BSL-2, Vero expressing Ebola virus VP30 for BSL-2/3

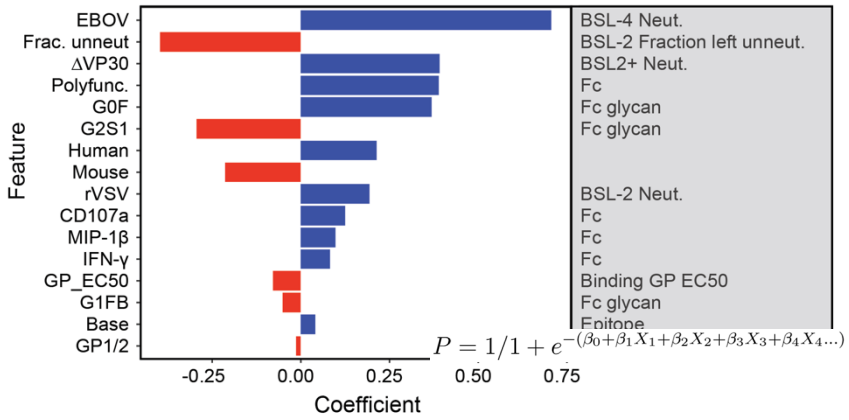


Figure 2. Coefficients of selected antibody features in a logistic regression model with elastic net regularization calculated by the above equation where *P* is probability and b is the coefficient that weights individual (x) features. Positive and negative coefficients indicate that an increase in the value of a given feature will increase and decrease, respectively, the probability of a mAb conferring protection in a mouse model of EBOV infection.

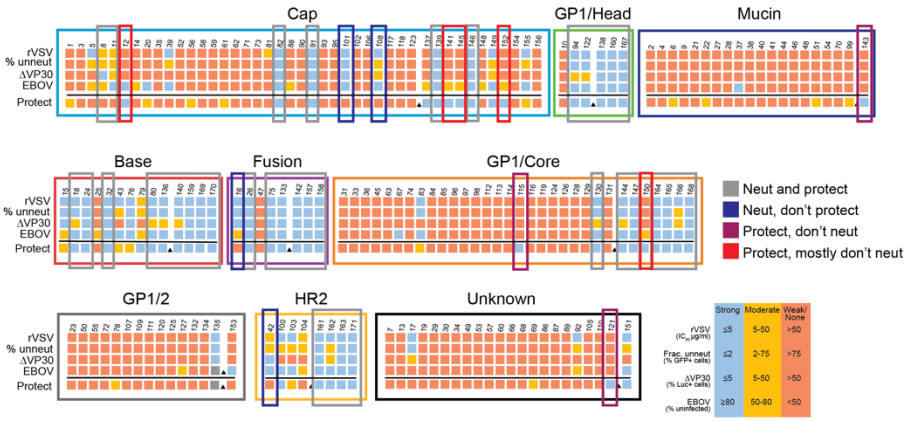
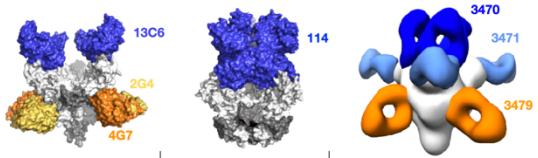


Figure 3. Relationship between in vivo protection and neutralization of rVSV (and fraction rVSV left unneutralized), replication deficient EBOV (ΔVP30) and authentic EBOV. The mAbs are grouped by epitope class. Boxes highlight mAbs that protected and neutralized, protected but did not neutralize, and protected but did not neutralize in all assay types.

and Vero E6 for BSL-4). There are several possible explanations for the greater predictive value of the BSL-4 and BSL-2/3 assays.

First, the glycoprotein presentation (e.g., spacing, density, glycosylation) on a filovirus-shaped particle could better reflect the presentation of authentic virus in challenge experiments than would its presentation on the bullet-shaped rVSV rhabdovirus particle. Second is the presence or absence of the secreted form of the glycoprotein, sGP, in the different assays. In Ebola virus infection, ≥80% of the viral glycoprotein transcripts drive production of sGP, a C-terminally truncated, secreted, soluble dimer (termed sGP), rather than the trimeric, transmembrane trimer, GP, which is required for viral entry[30]. Many antibodies cross-react with sGP and GP. These antibodies could interact with the abundant sGP and thus be unavailable to neutralize GP. The BSL-4 and BSL-2/3 assays contained wild-type sGP levels that are similar to expected levels in in vivo challenge experiments. In contrast, the rVSV vector was engineered to only produce GP and not sGP. As such, the performance of sGP-cross-reactive antibodies might be higher in the rVSV assay lacking distracting sGP than the BSL-4 or BSL-2/3 assays where sGP is present. Somewhat paradoxically however, sGP-cross-reactive antibodies were more likely to exhibit neutralization activity in the assays containing sGP. Indeed, 8/43 (nearly 20%) of the antibodies against the glycan cap, an epitope shared between sGP and GP, only exhibited neutralization against authentic Ebola virus, but these same antibodies showed no or low levels neutralization in either surrogate assay. Furthermore, 6/8 of these antibodies offered protection in the animal model.



	ZMapp Cocktail	mAb114 monotherapy	REGN-EB3 Cocktail
Source	Chimeric	Human	Human
Production	*Nicotiniana*	293F	Modified CHO
Survival (rhesus)	100%	100%	100%
Survival (humans)	50.3% (85/169)	64.9% (113/174)	103/155 (66.4%)

Figure 4. Antibody treatments tested for efficacy in 2018 outbreak of Ebola virus disease. In the PALM clinical trial, three 50 mg/kg doses of ZMapp were delivered on days 1, 4 and 7. A single 50 mg/kg dose of mAb114 was given on Day 1 and a single 200 mg/kg dose of REGN-EB3 was given on Day 1.

The importance of Fc-mediated protection: The VIC study indeed found that neutralization correlated with protection. However, two sets of interesting outliers contrasted with our expectation that neutralization would predict protection. One set of outliers exhibited potent neutralization activity but failed to protect *in vivo*. A second set had weak to no neutralization activity yet nonetheless offered protection *in vivo* (Figure 3). Analysis of the first set, the antibodies that neutralized potently but failed to protect, revealed that these antibodies largely lacked Fc effector functions like antibody-dependent phagocytosis or natural killer cell activity. In contrast, each antibody in the second dry that protected in the absence of neutralization exhibited multiple Fc effector functions. In our logistic regression analysis, the ability of the Fc to inspire Fc functions, particularly multiple Fc functions (i.e., Fc polyfunctionality) strongly correlated with protection. It is likely that such antibodies may be more common in the polyclonal

response to natural infection or vaccination. The strongest correlate of protection in many vaccine studies is simply antibody that binds the target antigen, and not neutralizing antibody[31–33]. In the VIC study, we also analyzed candidate therapeutics: antibodies that entered our study were chosen for donation by the labs that discovered them and downselected them. Thus, a bias toward neutralizing mAbs in samples sent for analysis could have existed simply because neutralization activity is straightforward to identify and select.

Typically, antibodies in a pool are downselected based upon their ability to neutralize and only those that neutralize are subsequently evaluated for *in vivo* protection. In the VIC study, however, all antibodies were evaluated for in vivo protection. A retrospective analysis of all antibodies in the study suggested that selection based on neutralization would have identified 60% of those antibodies that were ultimately protective. Selection based on Fc polyfunctionality also would have identified 60% of the protective antibodies. Sometimes these criteria would have identified the same antibodies (those that offer both neutralization and Fc functions), whereas other criteria would have identified different antibodies (those that protect solely by potent neutralization or those that protect solely by Fc or another non-neutralizing mechanism)[27]. The predictive value of Fc function in these studies suggests the importance of evaluating multiple antibody criteria to choose therapeutics or understand correlates of polyclonal protection.

Computational analysis of the broad dataset proposed that an ideal combination of antibody activities would be potent neutralization plus Fc polyfunctionality. The most potent neutralization occurred for antibodies binding the core of GP that remains after endosomal processing, or epitopes including the receptor-binding head, fusion loop, base and stalk. Fc polyfunctionality tended to include those epitopes on the upper and outer half of the molecule: the receptor-binding head, the glycan cap and in some cases the mucin-like domain. Note that the receptor-binding head exists at a possible “sweet spot” that allows both mechanical neutralization and recruitment of Fc functions (Figure 1). A limitation of the 2018 VIC study is that the mouse model was used for in vivo evaluation due to the need to characterize 168 mAbs. Analysis in non-human primates of antibody combinations predicted by computational analysis is underway. In the meantime, however, data for human clinical trials are available for three antibody preparations, and most of the antibody components of these preparations were analyzed in the VIC study. The PALM trial found that the two most effective treatments were REGN-EB3 and mAb114[34]. REGN-EB3 is a triple antibody cocktail that includes one antibody that neutralizes potently and two that appear to recruit Fc functions. mAb114 is a monotherapy that binds the receptor-binding site that is associated with both mechanisms of protection (Figure 4) [27,35,36]. The mortality for REGN-EB3 and mAb114 was significantly lower than that for an earlier cocktail, ZMapp[37] (p=0.0007 and p=0.0002, respectively)[34].

The evaluation by the VIC of a large array of antibodies contributed by different laboratories and compared using an range of standardized assays defined antibody features that are most closely associated with protection provided a framework to guide future antibody evaluation, and perhaps will allow discovery of antibodies that have activities outside of those that are most easily tested[38]. Outcomes of the VIC, and complementary efforts[26,27,35,36,39,40], indicate that discovery efforts that incorporate searches based on neutralization *and* Fc function would identify almost all (~>95%) of protective antibodies.

CORONAVIRUS SARS-CoV-2

An effort to compare therapeutic antibody candidates against SARS-CoV-2 is now in progress by the Coronavirus Immunotherapeutics Consortium (CoVIC). CoVIC is modeled on the VIC study for Ebola, and was launched in March 2020 by the Therapeutics Accelerator of the Bill & Melinda Gates Foundation, Mastercard, Welcome, the GHR Foundation and others, and expanded and extended by NIAID in July to include additional measures of escape, Fc activity and *in vivo* modeling. CoVIC will perform a side-by-side analysis of leading antibody candidates from an array of American and international contributors in a battery of *in vitro* and *in vivo* assays to measure pseudovirus neutralization, live virus neutralization, variables in neutralization, binding constants, escape propensity and location, Fc activities, high-resolution structural biology and *in vivo* protection in different animal models.

For therapeutic development, CoVIC will provide independent measurements of antibody activities and apple-to-apples-comparisons across a range of standardized assays. We also aim to identify antibodies of high potency and good manufacturing characteristics that can be mobilized to save lives in low- and middle-income countries. For fundamental research and in defense of seasonal return of coronaviruses, we further aim to establish a broad, publicly-accessible database of the landscape of antibody activities against SARS-CoV-2, escape vulnerabilities and antibodies that remain responsive to emerging mutations, as well as fundamental information on the quality and utility of Fc activities for *in vivo* protection and the link between animal model and human protection. Against this novel virus, and in 'Warp Speed' progress, we find ourselves in the unusual circumstance that we may have human clinical data in advance of many animal models and non-neutralization measures of activity. We thus hope to use the broad array of information to understand for the future to understand the predictive value of different animal models and *in vitro* analyses for human clinical benefit.

CoVIC assay set-up and performance are underway and we welcome feedback to ensure that the standardized assays performed for this consortium will provide long-lasting, durable information relevant for the research field (Figure 5).

Binding: Binding studies will be performed in a Good Laboratory Practice (GLP) setting, at Prof. Georgia Tomaras's laboratory at The Duke Human Vaccine Center. High-throughput surface plasmon resonance (SPR) using the Carterra LSA platform and biolayer interferometry (BLI) assays using the Forte Octet will assess binding kinetics to full-length spike, D614G and other variants that arise, as well as the association and dissociation rates for the receptor-binding domain (RBD) and N-terminal domain (NTD) will be performed. The ability of mAbs to block ACE2 binding will also be assessed, and high-resolution epitope binning of the range of therapeutic candidates will be carried out. Additional studies by Carterra can evaluate binding of the panel of mAbs to the body of monoclonal antibody resistance mutations (MARMs) generated against individual mAbs.

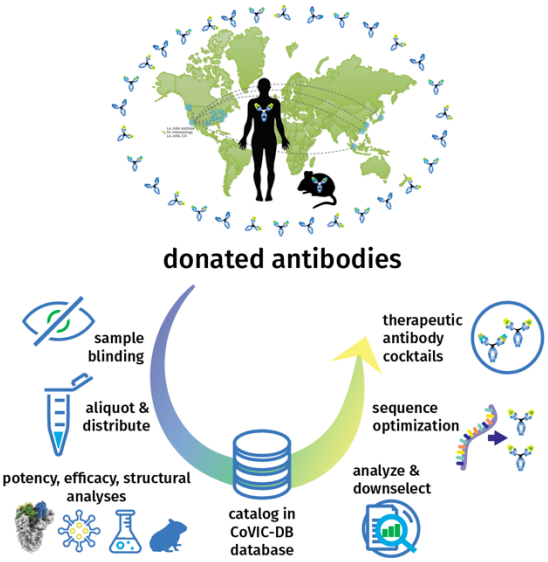


Figure 5. CoVIC workflow.

Neutralization: Neutralization capacity of mAbs contributed to the CoVIC will be evaluated using both pseudovirus and authentic virus systems, with additional studies to consider important biological variables. Pseudovirus neutralization assays for CoVIC have been qualified and will be performed by Nexelis based on a method described by Whitt (2010)[41] that uses genetically modified Vesicular Stomatitis Virus from which the glycoprotein G is removed (VSVΔG). VSVΔG virus is transduced in HEK293T cells previously transfected with SARS-CoV-2 coronavirus spike (Wuhan strain, accession NC_045512) lacking the last 19 amino acids of the cytoplasmic tail (ΔCT). The resulting pseudoparticles (VSVΔG-Spike ΔCT) contain a luciferase reporter to provide a signal that can be quantified in relative luminescence units (RLU). Neutralization activity will be assessed by 11-point concentration curves (mAb concentrations ranging from 0.004-3.6 µg/mL) from which IC_{50} and IC_{90} will be determined from four-parameter logistic curves. Other key variables, such as target cell type and presence or absence of TMPRSS2 will be explored at La Jolla Institute for Immunology (Saphire lab), beginning with a subset of antibodies that have a range of potencies and epitopes.

Neutralization of authentic SARS-CoV-2 will be performed in Alexander Bukreyev's lab at the University of Texas Medical Branch, Galveston (UTMB). Assay qualification at UTMB is in progress. Neutralization will

be assessed using virus engineered to express mNeonGreen (SARS-CoV-2-mNG)[42] for high-throughput measurement and a readout that is more consistent than that achieved with plaque reduction neutralization tests (PRNTs) and manual counting. Under BSL-3 containment, mAbs at a range of concentrations will be incubated with SARS-CoV-2mNG for one hour prior to application to Vero E6 cells pre-seeded in 96-well plates at a MOI=0.5. At 16 h post-infection, mNeonGreen-positive cells indicating infection will be quantified using a high-throughput imaging reader[43]. Neutralization curves will be generated, from which IC_{50} and IC_{90} values will be determined. Neutralization activities measured by PRNT and with high-throughput SARS-CoV-2mNG microneutralization assay were shown to be comparable (R^2=0.90).

Structural biology: Cryo-electron microscopy (cryo-EM), cryo-electron tomography (cryo-ET) or X-ray crystallography using recombinant spike, transmembrane-anchored spike, or RBD or NTD domains, respectively, will be performed by the Saphire laboratory at La Jolla Institute for Immunology using two dedicated 300 keV Titan electron microscopes with direct electron detectors and a Gatan energy filter, as well as an Aquilos instrument and cryo-correlative light and electron microscopy (cryo-CLEM) for visualization of structures in their biological, transmembrane context. Footprints, contact residues, stoichiometry, binding angle, adjustments to spike and synergy upon binding can be visualized with high-resolution structural biology. Our laboratory routinely does both cryoEM and X-ray for single particle structural analysis, with cryo-EM used to visualize complexes with trimeric spike and X-ray used to visualize complexes with RBD or NTD.

Escape and Surveillance: Key to durability of candidate therapeutics is their resistance to mutagenic escape and their ability to maintain neutralization of variant forms of the spike protein that may arise. Bette Korber's laboratory at Los Alamos National Laboratory will survey the GISAID database of coronavirus sequences for experimental evaluation of the ability of candidate therapeutics to remain responsive to different variants[44]. Specific escape mutation information will be generated by Yoshihiro Kawaoka's laboratory at the University of Wisconsin using rVSV and authentic SARS-CoV-2 with Vero E6 cells engineered to express TMPRSS2. Single mAbs and mAb combinations will be analyzed for escape propensity and location of escape mutations in the spike protein. mAbs will further be screened for binding and/or neutralization of other escape mutants. The goal is to identify a series of clinical candidates having differing susceptibilities, so that all candidates in use are not escaped by the same mutation and a library of antibody therapeutics can be maintained for use in multiple seasons.

Fc profiling, systems serology: The Alter Laboratory at the Ragon Institute of MGH, Harvard and MIT will profile the ability of each therapeutic antibody candidate to inspire a range of Fc-mediated activities. Functions profiled will include antibody-dependent cellular cytotoxicity, phagocytosis, activation and maturation of innate immune effector cells, cellular degradation, complement deposition, and antigen uptake of innate immune cells. Cells surveyed will include NK and dendritic cells, neutrophils, monocytes and macrophages, and both human and murine effector cells[45–47] for comprehensive capture of cross-species correlates with immunity against SARS-CoV-2[48-50]. These studies will capture about 50 data points for each mAb, and will be linked to structural and *in vivo* findings, using both univariate and multivariate tools, to define relationships between antibody effector profiles and epitope specificity and Fc features that track with protection against SARS-CoV-2.

Fc profiling, cellular studies: In Alexander Bukreyev's laboratory (UTMB), anonymized mAbs will be evaluated for the ability to induce innate immune effector function in a range of cellular assays. Assays will be performed with primary human myeloid cell populations (monocytes, macrophages, immature and mature dendritic cells (DCs) and NK cells) isolated from human donor blood by magnetic sorting[51,52]. We will evaluate the dependence of enhanced uptake on avidity of Fc domain–Fc receptor interactions, the effects of blocking each main type of Fcγ receptor on enhanced uptake[53], and will characterize the role of specific Fc effector functions[39,54]. Any links between isotype, epitope, neutralization capacity or other characteristics and enhanced uptake *in vitro* will also be established.

In vivo analysis: Efficacy of anonymized mAbs will be tested in Syrian golden hamsters and in mice expressing human ACE2 under control of the K18 promoter[55]. The Syrian golden hamster model is as previously described[56]. The novel mouse model is lethal by day 5. For therapeutic administration, Syrian

golden hamsters or K18h-ACE2 mice, each 4-6 weeks old, will be intranasally inoculated with $2x10^5$ PFU/animal SARS-CoV-2. We plan to deliver prophylactic antibody at 10 or 20 mg/kg 12 hours after challenge, although a dose finding study is in progress to evaluate other data points. Nasal turbinates and lungs will be collected and evaluated on days 2, 4 and 14. A pre-exposure prophylactic dose-finding study is also planned.

In a separate *in vivo* study at a separate location, Sujan Shresta's laboratory at La Jolla Institute for Immunology, we will evaluate a novel COVID-19 mouse model that expresses human ACE2, human FcRn and human TMPRSS2 using anonymized mAbs. As an animal model of COVID-19, these mice could better recapitulate mAb pharmacokinetics (PK), and potential risk factors for ADE. The clinical success of a therapeutic CoVIC mAb can be tied to longevity (half-life), a feature related to its affinity for the FcRn receptor, which is expressed on endothelial cell membranes and constantly endocytoses IgG from the plasma and recycles it back into the plasma. Mouse FcRn has 10-fold higher affinity for human IgG than human FcRn, such that human mAbs have extended half-lives in mice. Thus, mouse models provide comparatively poor representation of human pharmacokinetics[57–61] and can complicate modeling of Ab half-lives, virus neutralization, and likelihood of therapeutic Ab-FcRn interactions and immune complex formation. mAbs engineered to have minimal Fc receptor binding (e.g., LALA mutant antibodies) have normal human FcRn binding sites and interactions[62], whereas Abs engineered to have extended half-life (e.g., YTE, LS, Xencor Xtend mutations) have enhanced FcRn affinity. FcRn may also play a role in endothelial cell uptake of immune-complexed SARS-CoV-2 virions. For Dengue, ADE can be abrogated by a strong CD8 T cell response[63]. FcRn promotes presentation of viral antigens to CD4 and CD8 T cells[64,65], thereby bridging humoral and cellular immunity.

CoVIC Database "CoVIC-DB": Anonymized results from all the assays will be made publicly available in the CoVIC database, developed by Bjoern Peters's laboratory at La Jolla Institute for Immunology. For the neutralization assays in particular, templates for data upload will be standardized so that comparisons can easily be made between antibody performance against authentic virus and pseudovirus platforms. The assays will be benchmarked against a standardized set of neutralizing antibodies currently being produced at scale by LakePharma for delivery to all partner reference labs. Analysis of anonymized antibody samples in the database will allow better understanding of the landscape of antibody activities against SARS-CoV2, including which Fc functions predominate at which epitopes sites, which characteristics lead to most potent neutralization, which potential escape mutations impair which classes of antibodies, etc. Antibody contributors will know which samples in the database are theirs and will have the opportunity to contact the Program Manager, Dr. Sharon Schendel, should a particular datapoint not match expectations and thus needs to be re-run or re-analyzed. The purpose and goal of CoVIC is to facilitate acceleration of antibodies to the clinic by providing complete, complementary and independent analysis in standardized assays. It is our hope that the broad participation in CoVIC, by both antibody developers and partner analysis labs alike, will together build an arsenal of needed therapeutics and a broad and deep database of information we may use to respond to SARS-CoV-2 this year, the coming seasons, and against future emerging threats.

SELECTION OF ANTIBODY COMBINATIONS

Using the database of the VIC study, we ranked the antibody characteristics measured *in vitro* for their correlation with protection. We next generated a multi-variable model to predict protection of VIC mAbs alone and in combination. Computational analysis of this model identified combinations of eight key features (fraction unneutralized pseudovirus, neutralization of authentic EBOV, Fc polyfunctionality, several glycan structures and IFN-γ release) that were most predictive of *in vivo* protection. We predicted two cocktails, one EBOV-specific, and the other cross-reactive with other ebolaviruses (Sudan and Bundibugyo) that comprised two and three antibodies, respectively. As predicted by a generalized linear model, each antibody cocktail maximized neutralization, immune effector function and other protective features over the individual antibodies alone [25]. Evaluation of these predicted cocktails in nonhuman

primate models is in progress. In the meantime, human clinical data exists from another antibody cocktail selected with an eye on similar complementary routes to protection.

The Ebola virus cocktail that offered the greatest protection in human clinical trials[34] was developed at Regeneron by considering several features: neutralization of pseudovirus, recognition of the receptor-binding core of GP at the relevant endosomal pH to block receptor binding and membrane fusion, binding to sGP, and elicitation of Fc effector functions to promote killing of EBOV-infected cells[35]. Three antibodies that fulfilled these criteria and did not cross-compete were selected to form the triple cocktail REGN-EB3 (Figure 4), which showed a superior reduction in mortality in humans with Ebola virus disease [34].

A similar strategy was used to identify an antibody cocktail for SARS-CoV-2 with lessons that are informative for other cocktail selection efforts[66]. In this study, 40 antibodies with unique sequences and potent, picomolar neutralization activity in a VSV pseudovirus assay were initially selected from a panel of >200. Interestingly, this screen identified other antibodies that also had affinity for the spike protein of the original SARS virus, but these cross-reactive antibodies had low neutralization activity. This finding is consistent with that for antibodies against ebolaviruses in that cross-reactivity appeared to come at the expense of neutralization and in vivo protection [25].

The 40 candidates were further downselected to 9 that had the highest neutralization potencies (7 pM-99 pM), and the ability to bind SARS-CoV-2 RBD and block interactions with ACE2 at double-digit pM IC_{50} values. Focusing on 4 candidates that all had pM affinity for trimeric SARS-CoV-2 spike, neutralization against authentic SARS-CoV-2 at BSL-3, and neutralization of different pseudovirus particles either bearing spike with a monobasic cleavage site or a deletion of the furin-cleavage site. Notably, infectivity of pseudoparticles bearing stabilized spike was similar to wild type spike that retained the cleavage site in Vero cells, but there was substantial loss of infectivity of Calu-3 cells, a lung epithelial cell line. This result suggests that involvement of proteases may vary between cell lines and should be considered when interpreting neutralization assay outcomes[67].

In cross-competition binding assays, several pairs of non-competing mAbs were identified. Hydrogen-Deuterium Exchange Mass Spectrometry defined the contacts for these mAbs on the RBD surface and helped reveal possible pairings of mAbs in therapeutic antibody cocktails. Two in particular, REGN10933 and REGN10987, bound distinct regions on the RBD: from the top of the RBD and from the lower left side, respectively. REGN10987 is predicted to block ACE2 binding, whereas the predicted epitope for REGN10933 shows little overlap with the ACE2 binding site.

In a separate cocktail-selection study, other monoclonal antibodies against SARS-CoV-2 were selected from a large panel[68] by their performance in a quantitative focus reduction neutralization test (FRNT) with SARS-CoV-2 strain WA1/2020[69]. Next, biolayer interferometry was used to sort antibodies into competition groups. Among non-competitive, potently neutralizing antibodies, COV2-2196 plus COV2-2130 demonstrated synergistic neutralization in that 79 ng/mL of the cocktail (16 ng/mL and 63 ng/mL COV2-2196 and COV2-2130, respectively) had the same activity as 250 ng/mL of each individual mAb. These two mAbs bound different epitopes within the spike RBD and also recognized different conformation states of trimeric spike. COV2-2130 appeared to recognize the RBD in both the “open” and “closed” conformations, whereas COV2-2196 tended to recognize the “open” conformation in which the RBD is rotated upward, exposing residues that are involved in interactions with ACE2. This synergy suggests that through selection of optimal cocktails, the dose of each mAb can be reduced. The cocktail showed protection in two mouse models of SARS-CoV-2 infection as well as in NHPs infected with SARS-CoV-2.

Taken together, these studies show how neutralization assays can form the basis for selection of highly potent antibodies, that can then be downselected based on antigen-binding affinity, structural analyses of binding footprints, and other features to select ideal cocktails that maximize antigen coverage and reduce the likelihood of escape. Additional cocktails will be selected by CoVIC for mobilization to low- and middle-income countries based on similar criteria: neutralization potency across a range of assays and cell types, complementary binding footprints as revealed by structural biology, and resistance to

escape, while considering Fc functions consistent with those that provide protection in human clinical studies of vaccine-mediated protection and passive administration with convalescent sera.

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CONCLUSION

Key takeaways from each session

Dr. Collins concluded the meeting by highlighting key points from each session.

- Randomized clinical trials with convalescent plasma must continue, and Covid-19 survivors should be encouraged to continue donation of plasma. The lack of adverse events with this therapy to date is reassuring and indicates that Antibody-Dependent Enhancement (ADE) is unlikely to be a problem for neutralizing antibody therapies. Clinical monitoring will be important for signs of immune complex disease or unusual manifestations of the disease.
- Resistance to antibody therapies is a significant risk, and intense monitoring during trials will be needed to see if resistance is developing. Cocktails of multiple antibodies may reduce the risk of escape, but provide challenges logistically and in regulatory review. We need to develop and support reliable *in vitro* assays for predicting synergism among antibodies in cocktails.
- While an impressive toolbox of Fc modifications has been used successfully to increase half life of antibodies and modify effector function for other therapies, this has not been widely used for infectious diseases. More research is needed on the natural clearance of SARS-Co-V2 before antibodies can be engineered in an informed way. We need to understand the role of IgA, but manufacturing hurdles to IgA therapies would be substantial. Analysis of bispecific antibodies versus cocktails of two antibodies needs careful consideration.
- The chief lesson learned from neutralizing antibody therapies for other conditions is that antibody potency is the key factor in efficacy. While cocktails of antibodies may help prevent resistance, a single potent neutralizing antibody against Ebola was as effective as a three-antibody cocktail.
- Standardized assays and data are critical. We need to learn all we can from clinical trials, and data standards will be important. Blinded analysis of antibodies is available through the Coronavirus Immunotherapy Consortium (CoVIC). We need to apply multiple different assays and compare results with in vivo neutralizing activity. These analyses will enable the most potent antibodies to be identified and their quality to be assessed through the manufacturing process. Standard assays may also be useful for identifying synergistic cocktails.

After a final appeal for every neutralizing antibody to be submitted to CoVIC for standardized assays as soon as possible, Dr. Collins ended the meeting with a call for everyone to continue pressing forward for an effective treatment, saying “the world is waiting.”

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