A counterintuitive antibody cocktail disrupts coxsackievirus

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Utilizing monoclonal antibodies to prevent and treat infectious diseases has been accelerated by the COVID-19 pandemic. In this issue of *Cell Host & Microbe*, Zheng et al. show how a three-monoclonal-antibody cocktail, that defies conventions of "rational design" for a therapeutic agent, functions cooperatively to disrupt coxsackievirus virions.

While monoclonal antibody (mAb) technology is over 40 years old, for more than two decades, the only antiviral mAb therapy licensed by the FDA was palivizumab (Johnson et al., 1997), for pre-exposure prophylaxis to protect high-risk infants from hospitalization due to respiratory syncytial virus infection. Efforts to apply mAbs to treat, rather than prevent, viral infections were finally successful for Ebola virus (Mulangu et al., 2019), leading to FDA licensure in 2020. As human mAbs have become easier to isolate and characterize at ever-larger scales, mAbs have greatly accelerated our understanding of antibody-mediated antiviral immunity and have given us new prophylactic and therapeutic options for viral diseases. The SARS-CoV-2 pandemic is an especially dramatic example, during which potently neutralizing human mAbs were isolated from survivors in March and April of 2020 and were in clinical use before the end of the year (Chen et al., 2021).

A potential limitation of using mAbs as antiviral agents to target RNA viruses is that resistance could emerge as population immunity drives the selection of antigenic variants. One approach to mitigating this risk is to use multiple mAbs in combination to target different epitopes, because it is less likely for a virus to simultaneously acquire multiple amino acid substitutions. This concept has been extensively explored *in vitro* for SARS-CoV-2 (Baum et al., 2020). Another advantage of mAb combinations is redundancy—if a mutation that confers resistance to one mAb emerges, the other mAb(s) in the combination can still bind and neutralize, preserving overall activity.

This was the case for the SARS-CoV-2 Delta variant, against which the mAb bamlanivimab lost neutralizing activity but the combination of bamlanivimab and etesevimab retained activity (Planas et al., 2021).

In some cases, the activity of mAb combinations can also be greater than the sum of their parts, a phenomenon known as synergy. For mAbs targeting Ebola virus, mAb cocktails have been described where one mAb enhances the binding of another mAb to a cryptic epitope, potentiating neutralization and providing enhanced protection *in vivo* (Gilchuk et al., 2021). A combination of two SARS-CoV-2 mAbs, tixagevimab and cilgavimab, has been authorized for pre-exposure prophylaxis in immunocompromised individuals. These mAbs are able to recognize different conformational states of the SARS-CoV-2 spike trimer and have been shown to synergistically neutralize the virus (Zost et al., 2020). Importantly, though the emergence of the highly mutated Omicron BA.1 variant reduced the neutralization potency of each individual mAb, the combination of tixagevimab and cilgavimab was better at neutralizing Omicron BA.1 than either mAb alone (VanBlargan et al., 2022).

Increased resistance to escape, functional redundancy, and synergy between components are all desirable features of mAb cocktails. But what is the best way to go from isolating individual monoclonal antibodies to figuring out which combinations function best together? In this issue, Zheng and colleagues present an in-depth mechanistic investigation of a combination of three neutralizing mAbs that all compete with the coxsackievirus-adenovirus receptor (CAR) for binding to coxsackievirus B1 (CVB1) (Zheng et al., 2022). The authors selected murine mAbs that disrupted the binding of recombinant CAR to CVB1 virions in an ELISA. Three of the mAbs neutralized the virus potently *in vitro* (with half-maximal neutralizing concentrations 376 ng/mL or lower), bound well to mature virions (with sub-micromolar affinity), and protected mice from death in a lethal model of CVB1 infection (1 mg/kg or less provided full protection). These properties were similar when the mAbs were generated recombinantly as chimeric IgG molecules with human fragment crystallizable (Fc) regions and the native murine fragment antigen-binding (Fab) regions.

Intriguingly, when the investigators explored combinations of the three chimeric mAbs, both in pairs and as a trio, they noted synergy both *in vitro* and *in vivo*. Synergy is often incorrectly used to describe mAb cocktails in which combined antibodies simply neutralize to the potency of the strongest mAb in a cocktail. In this manuscript, though, the authors mixed equimolar concentrations of mAbs in their cocktails and observed *in vitro* neutralization and *in vivo* protection to levels better than would be expected if considering the concentration of the most potent mAb in the cocktail alone, which was a true observation of synergy.

Although demonstrating synergy can be difficult, describing mechanisms that drive synergy is an even taller order. However, Zheng and colleagues were up to the task, combining biochemical methods and solving 22 different structures of CVB1 in complexes with Fab regions of

the mAbs. All individual Fabs were able to displace pre-bound CAR, and the epitopes of all three Fabs overlap to varying degrees. If the mAbs neutralized only by interfering with virion:receptor engagement, we would not expect to see synergy in cocktails of the mAbs because they are all simply competing with each other and with CAR for binding to the virion—the highest affinity interaction would simply win and drive the experimentally observed efficacy because of this single mechanism of neutralization. So the authors' observation of synergy implied that their cocktails likely have an additional, cooperative neutralization mechanism in which binding of one mAb potentiates better or additional function of another mAb.

Though the authors identified structures of multiple populations of Fab:virion complexes by mixing different pairs of Fabs with CVB1 virions, the structures of the three-Fab combination mixed with CVB1 yielded the most compelling observations. While 90% of the structures observed appeared to be homogeneous virions occupied by the highest affinity Fab, upon focusing symmetry constraints on the 5-fold axis of symmetry rather than the Fabs, the authors noted the presence of virions bound by heterogeneous mixtures of two different Fabs. So steric hindrance from this highest affinity Fab was not sufficient to block all binding of the other Fabs. More surprisingly, the overall number of Fab:virion complexes observed was substantially diminished; it appeared as if the three-antibody cocktail caused a portion of the virions to disintegrate entirely. Proving the absence of an entity is difficult, so to further investigate this possibility, the authors also examined virus preparations treated with the three-Fab combination using high-performance size-exclusion chromatography and noted a drop in area under the curve of structured protein after antibody treatment. This supported the hypothesis that the three-Fab cocktail was destroying virions.

Observations suggesting that mAb combination antiviral therapies may be capable of destroying a virus on contact are exciting for medicine but also push science toward a better understanding of polyclonal mechanisms of neutraliza-

tion. Though most structural studies to date focus on one or two Fabs in complex with virions, by increasing to even just a third Fab, the authors uncovered a striking mechanism of neutralization. Of course, true polyclonal antibody responses are much more complex, so increasingly complex structural studies like this one are needed to further understand neutralization *in vivo*. Importantly, Zheng and colleagues' reductionist approaches to dissecting how three mAbs disrupt CVB1 provide a roadmap for further studies examining how synergistic neutralization occurs in the polyclonal antibody responses that individuals make to infection or vaccination, which could also inform vaccine design.

We have previously isolated mAbs that bind to enterovirus D68, a member of the *Enterovirus* genus like CVB1, and showed that these mAbs can be binned into at least 5 "competition groups" (Vogt et al., 2020). Typically, rational design of therapeutic mAb cocktails to prevent escape mutations while maximizing antibody occupancy of the virion (by limiting the possibility of steric interference between mAbs) would dictate selecting non-competing mAbs. However, Zheng and colleagues have elegantly demonstrated that this approach would miss potential synergistic mAb combinations that may be highly efficacious *in vivo*. A challenge for the field is to further develop empiric approaches to find synergistic mAb combinations rather than relying on conventional wisdom. As the authors have shown, that will require marrying disciplines from structural biology to virology to immunology. In both scientific research as well as synergistic mAb combinations, it seems that teamwork makes the dream work.

DECLARATION OF INTERESTS

M.R.V. is a paid consultant for IDBiologics, Inc. and is co-inventor on a patent application for anti-enterovirus D68 human monoclonal antibodies filed by Vanderbilt University. S.J.Z. is a co-inventor on patent applications for anti-SARS-CoV-2 human monoclonal antibodies filed by Vanderbilt University.

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