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Antibody and Effector Cells Collaborate to Reduce SARS-CoV-2 Yield from Infected Cells

THESIS

Submitted in partial satisfaction of the requirements

for the degree of

MASTER OF SCIENCE

in Biomedical and Translational Science

by

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DEDICATION

To my husband, Nathan, thank you for believing in me and helping me to achieve my dreams. You are the best husband and partner I could have asked for. Your unwavering support and encouragement mean more than you could ever know. You put my needs above yours to help me further my career and I can't wait to see you earn your next degree. I'm so excited to see everything we can accomplish together. You are my favorite person, and I am so proud to call you my partner.

To my mom, Alexis, thank you for your words of encouragement, shoulder to cry on, and unconditional love. I am so thankful to have watched you grow into the remarkable person you are today, and I feel blessed to be able to grow beside you as your mini-me. I would not be where I am today without you.

To my dad, thank you for coming into my life and choosing me as your daughter. You are better than any dad I could have ever dreamt of…I expect extra kudos for this.

To my family and friends, thank you for always being by my side in the worst and best of times. I love you all and appreciate everything you have done to help me further my career and achieve my dreams.

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LIST OF ABBREVIATIONS

mAB	: Monoclonal antibody
SARS-CoV-2	: Severe Acute Respiratory Corona Virus- 2
SARS-CoV-1	: Severe Acute Respiratory Corona Virus- 1
COVID-19	: SARS-CoV-2 infection disease
-S	: Spike protein
S1	: Spike protein subunit-1
S2	: Spike protein subunit-2
RBD	: Receptor binding domain
ACE2	: Angiotensin-converting enzyme-2
RNA	: Ribonucleic acid
AT2	: Alveolar type 2
ARDS	: Acute Respiratory Distress Syndrome
Ig-	: Immunoglobulin
Anti-	: Antibody against protein
FcγR	: Fc gamma receptors
ADCC	: Antibody-dependent cellular cytotoxicity
ADCP	: Antibody-dependent cellular phagocytosis
NK	: Natural killer cell
COVA1-18	: IgG-S monoclonal neutralizaing antibody
ADCVI	: Antibody-dependent cell-mediated viral inhibition
BSL-3	: Biosafety level 3
PBMC	: Peripheral blood mononuclear cells
DEN3	: Dengue virus 3 IgG antibody
DMEM	: Dulbecco's modified eagle medium
P/S	: Penicillin-Streptomycin

FBS	: Fetal bovine serum
PBS	: Phosphate-buffered saline
MOI	: Multiplicity of Infection
PFA	: Paraformaldehyde
qPCR	: Quantitative polymerase chain reaction
FFA	: Focus forming assay.
SD	: Standard deviation
n	: Sample size
IC50	: Half-maximal inhibitory concentration

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ABSTRACT OF THE THESIS

Antibody and Effector Cells Collaborate to Reduce SARS-CoV-2 Yield from Infected Cells

By

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Master of Sciences in Biomedical and Translational Sciences

University of California, Irvine, 2023

Professor Donald Forthal, Chair

Antibodies against the SARS-CoV-2 envelope spike protein (S) are foundational for targeted therapies to reduce severity of COVID-19. Antibodies can neutralize extracellular virus and can potentially mediate other functions to inhibit virus. In this study, we determined the impact of primary human monocytes combined with COVA1-18, a SARS-CoV-2 neutralizing monoclonal antibody(mAB), on the yield of SARS-CoV-2 from infected cells. We found that antibody combined with monocytes resulted in lower viral yield than antibodies alone. Our findings suggest that enhancing interactions between mAB's and monocytes may improve the efficacy of mAB therapy for COVID-19.

CHAPTER 1: INTRODUCTION

Antibodies elicited by viral infections can neutralize extra-cellular viruses or kill virus-infected cells by engaging effector cells, such as monocytes or natural killer cells. Here, we hypothesize that monocytes in conjunction with neutralizing monoclonal antibody (mAB) will reduce the yield of SARS-CoV-2 from infected cells more than mAB alone. This study aims to determine if an anti-S-neutralizing mAB can engage with monocytes to produce a more potent anti-viral effect.

CHAPTER 2: BACKGROUND

In early 2020, Severe Acute Respiratory Corona Viruses (SARS-CoV-2), originating from Wuhan China in late 2019, entered the United States. This novel virus is a relative of the SARS-CoV-1 virus which made its way around the world in 2003 **(1)**. With over 70% genetic similarities between the two SARS viruses, both viruses cause flu-like symptoms and respiratory illness **(1,2)**. As of August 9th, 2023, there have been 769,369,823 confirmed COVID-19 cases and 6,954,336 deaths, bringing the total death rate to roughly 1% of the confirmed infected population. In the United States, there have been approximately 103,436,829 confirmed cases and 1,127,152 deaths, bringing the death rate in the United States to roughly 1.1% **(2)**. Although the death rate is considered low, more people likely had a SARS-CoV-2 infection who either did not test, or tested at home and did not report it. Given these two limitations, it is likely that the rate of infection is much higher than the rate calculated by the WHO **(2,3)**.

SARS-CoV-2 is primarily transported through aerosols produced by respiratory secretions released when an infected person talks, coughs, or sneezes and enters the body through the

mucous membranes located in the oral and nasopharynx **(1-3)**. Virus in aerosols can travel up to six feet from the infected person to a non-infected person **(1-3)**. The amount of virus needed to infect a human (infectious dose) is between 36 and 179 viral particles, based on non-human primate studies, **(4)**.

In most COVID-19 cases, mild cold and flu-like symptoms include cough, sore throat, nausea/ gastrointestinal upset, shortness of breath, headache, fatigue, and fever/chills; loss of taste and smell have also been recorded with multiple variants **(1-3,5)**. Of the confirmed cases, roughly 30% are reported to be asymptomatic, and 49% report mild to moderate disease **(5,6)**. 21% require hospitalization due to severe disease characterized by pneumonia, which can lead to acute respiratory distress syndrome and death **(7,8)**. An average of 6% of those hospitalized are admitted to the ICU, with admittance rates higher in persons with one or more pre-existing conditions (13%) **(7)**.

CHAPTER 2.1: SARS-CoV-2 Pathogenesis

SARS-CoV-2 binds to human cells via its spike glycoprotein protein (S) displayed on the virus's surface. The S-protein consists of two subunits, S1 and S2 **(9,10)**. The S1 subunit contains the receptor binding domain (RBD) which attaches to human angiotensin-converting enzyme-2 (ACE2) on the surface of susceptible cells **(9,10)**. The S2 subunit is responsible for membrane fusion between the virus and the cell. Once fusion occurs, viral RNA is delivered into the cytoplasm, and replication of the new virus begins. The newly formed viruses are then released via exocytosis to continue the replication process **(FIGURE 1) (14)**.

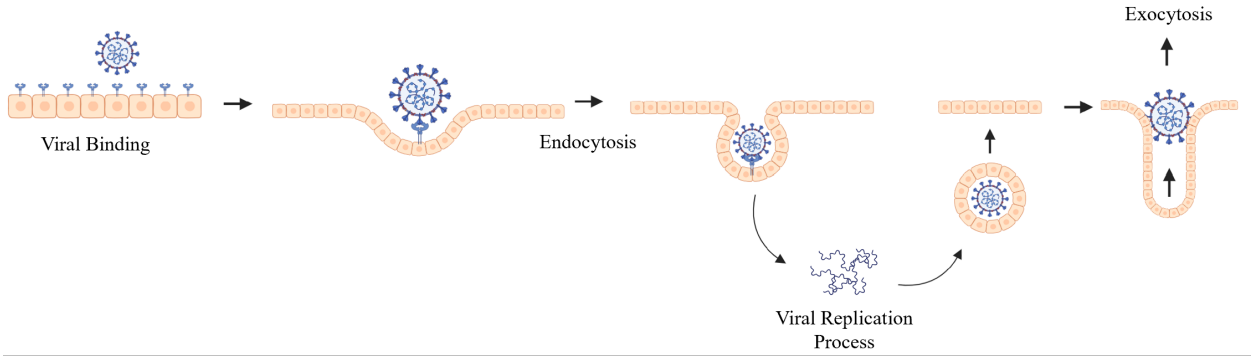

FIGURE 1: SARS-CoV-2 Replication Process. The spike protein binds to ACE2 on the surface of the host cells, after which, the virus membrane fuses with the cell membrane. The virus then replicates inside the host cell and the newly replicated virus leaves the host cell through exocytosis.

Infection begins in the upper respiratory tract and disseminates to the lower respiratory tract, infecting the alveolar type 2 (AT2) cells **(9-11)**. This may lead to pneumonia and acute respiratory distress syndrome (ARDS) **(9-11)**, with consequent impaired gas exchange and low blood oxygen concentration.

CHAPTER 2.2: Antibody Responses and Function During Infection

SARS-CoV-2-specific immunoglobulin (Ig) G, M, and A antibody subclasses develop in response to infection **(12-15)**. The role of antibodies in resolving infection is unknown, but it is clear that anti-S antibodies elicited by infection or by vaccines play a role in preventing subsequent infection. Moreover, S-specific monoclonal antibody obtained from recovered individuals effectively treats and prevents infection.

Antibodies can function in multiple ways, either through direct neutralization of the virus or by interacting with effector cells **(15,18,19)**. In the case of SARS-CoV-2, neutralization requires the antibody to bind the spike proteins on the virus's surface **(12-15,18,19)**. This interaction can block the spike proteins from binding to the host cells' ACE2, thus neutralizing the infectivity of

the virus **(12-14,18-21)**. In addition to neutralizing cell-free virus, IgG antibodies can inhibit virus yield from infected cells. This occurs when an antibody bound to the infected cells also binds to adjacent effector cells such as natural killer (NK) cells, monocytes, macrophages, or dendritic cells through Fc gamma receptors (FcγRs) on the surface of the effector cells **(19-23)**. The engagement of FcγRs results in antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP) **(23-40)**. Compared to NK cell-mediated ADCC, little is published on antibody-mediated effector functions of primary monocytes in SARS-CoV-2 infection **(37,38,41,42)**.

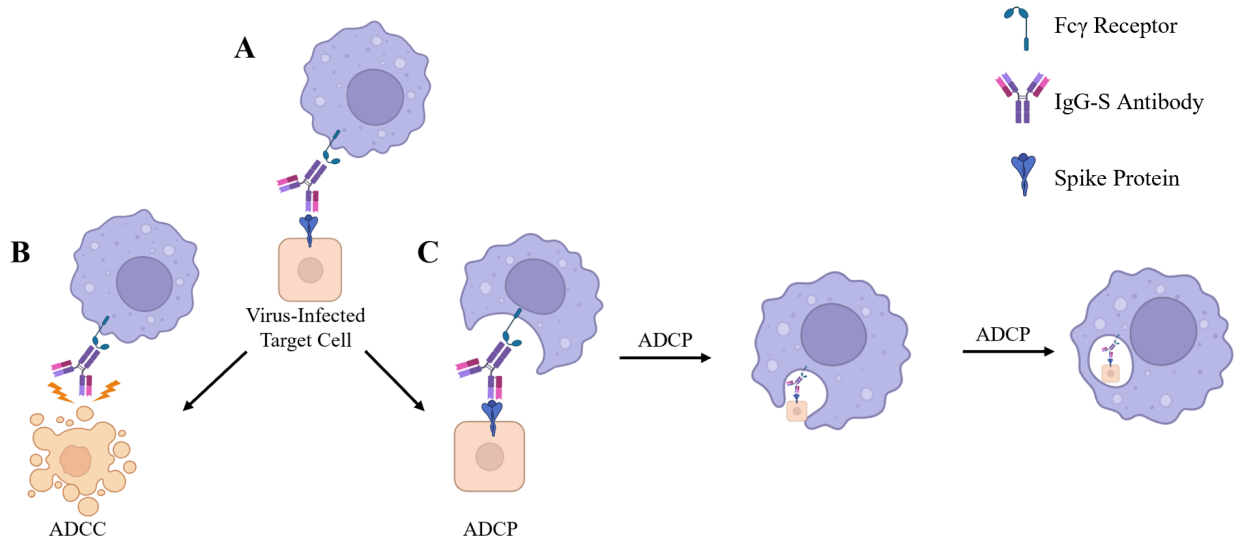

FIGURE 2: FcγR mediated inhibition of virus infection. A) Antibody bound to a virus-infected target cell via the Fab fragment and FcγR on an effector cell via the Fc fragment of the antibody. **B)** Antibody-dependent cell cytotoxicity (ADCC) results in target cell death. **C)** Antibody-dependent cell-mediated phagocytosis (ADCP) results in the internalization of the infected cell.

Since neutralization acts only on cell-free virus, antibody activities that impact the cells that produce the virus could have a more significant overall anti-viral effect. We sought to determine the effect of antibody combined with FcγR-bearing effector cells on reducing viral yield from SARS-CoV-2 infected cells.

CHAPTER 3: METHODS

All work including live SARS-CoV-2 virus was completed in the Biosafety Level 3 (BSL-3) facility at the University of California, Irvine.

CHAPTER 3.1: Viral Propagation

The Washington strain SARS-CoV-2, obtained from Microbiologics (Live SARS-2 WA-1/2020), was used for virus propagation. SARS-CoV-2 was grown using VeroE6 cells in tissue culture flasks (10e6 cells in a T-75 flask) at 70-80% confluency. 100uL virus was added to the flask with 1.9mL of media (DMEM+ P/S+L-Glutamine) without FBS and incubated at 37℃ with 5% CO2 for 45 minutes. After 45 minutes, 5mL media was added to the plate and placed in the incubator for 48 hours. The virus-containing supernatant was harvested and cleared from cell debris by centrifugation. Aliquots of the virus were stored at -80℃. A focus-forming assay (FFA) was used to determine the infectious viral titer. 15uL virus was added in a 1:10 dilution to a 96-well plate of VeroE6 (10,000 cells/well) with 35uL media. A serial dilution was completed where the virus was placed into the first well, resuspended five times, and then placed into the well below with 35uL media. This was repeated until a dilution of 1:10^-8 was achieved. The plate was incubated for 45 minutes before 1%MTC in DMEM+P/S+L-glutamine with a total percentage of 10% FBS. The plate was incubated at 37℃ and 5% CO2 for 24 hours before media was aspirated, and all cells were fixed with 4% paraformaldehyde. The cells were then permeated using methanol and hydrogen peroxide before being blocked in 5% non-fat dry milk in PBS for one hour. The cells were incubated with primary antibody overnight at 4℃, washed, and incubated with secondary antibody for 2 hours before being stained with KPL TrueBlue™

Peroxidase that binds to plaques to quantify visually. Once stained, the cells are dried for 24 hours before being taken to the ImmunoSpot plate reader and analyzed by visually counting and recording plaques. Plaques were counted at each serial dilution, and the titer was calculated using the equation: pfu (plaque forming units)/mL= ((Avg. plaques x dilution)/volume)x 1000.

CHAPTER 3.2: Neutralization Assay

The antibody of interest for this study is the COVA1-18 anti-S neutralizing IgG mAB. VeroE6 cells were used to test the neutralization of the S specific mAB, COVA1-18. DEN3, a Dengue virus 3-specific mAB was used as a negative control. Antibodies were placed into a plate at concentrations of 50ug, 10ug, 2ug, 0.4ug, 0.08ug, and 0.016ug. All concentrations were run in triplicate in addition to virus-only control with no antibody present. COVA1-18 was incubated with Washington strain SARS-CoV-2 at an MOI of 0.062 for 45 minutes before being added to VeroE6 cells. The cells were incubated with COVA1-18 and virus supernatant for 24 hours before being fixed with 4% paraformaldehyde (PFA), incubated overnight, and processed using an FFA. Neutralization was calculated using the following equation: Neutralization =100-(# plaques obtained with a given antibody concentration/# plaques with virus only) x100. A curve was fitted to the calculated percent neutralization (**FIGURE 5**) where a line marks the 50% inhibitory concentration (IC50). Using the neutralization curve, it was decided that 10 ug/mL would be used for our studies, due to the higher percent neutralization.

CHAPTER 3.3: Donor Cell Isolation

For this experiment, PBMCs were frozen after isolation from each donor and subsequently thawed before use, where monocytes were then isolated. Four participants (n=4) donated blood

to the University of California, Irvine Translational Science division. All donors were confirmed to have no known immune disorders during donation. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors using a Lymphoprep™ density gradient medium (Stemcell Technologies) according to the manufacturer's instructions. PBMCs were counted, resuspended in a freezing medium (i.e., 90% FBS and 10% DMSO), aliquoted, and stored at -80*C for further applications. For primary monocyte isolation, PBMCs were thawed, washed, and resuspended in a monocyte isolation buffer (2% FBS and 1 mM EDTA in DPBS). Untouched monocytes (CD14++CD16-) were isolated with a classical monocyte isolation kit from Miltenyi Biotec according to the manufacturer's instructions. Cells were counted and seeded in the respective cell numbers used for the assays.

CHAPTER 3.4: ADCVI Assay

VeroE6 cells were infected with Washington strain SARS-CoV-2 virus at an MOI of 0.062 for 45 minutes and washed with PBS before adding the antibody and monocyte effector cells to the infected target cells. Multiple concentrations of the COVA1-18 mAB were used, both alone and with monocyte effector cells. Controls for this experiment included target cells infected with virus only (positive control), and monocytes without mAB in the presence of virus-infected target cells. All conditions were repeated with a negative control antibody (DEN3). Monocytes were added at an effector cell: target cell ratio of 5:1 (50,000 monocytes added to 10,000 infected VeroE6 cells). Additionally, all conditions were run in triplicate for each donor. After a 72-hour incubation period, the supernatant was collected from the plates where 90uL was inactivated using ZYMO™ DNA/RNA shield (1:1 ratio of reagent and SARS infected media), and 90uL was frozen at -20C. The remaining cells were washed with PBS, and 200uL of DMEM

Media without FBS was placed in the -80 freezer to lyse the cells. The lysed cells/media were thawed, and 90uL was collected and inactivated using the ZYMO^{TM} DNA/RNA shield for qPCR to quantify viral load for each condition.

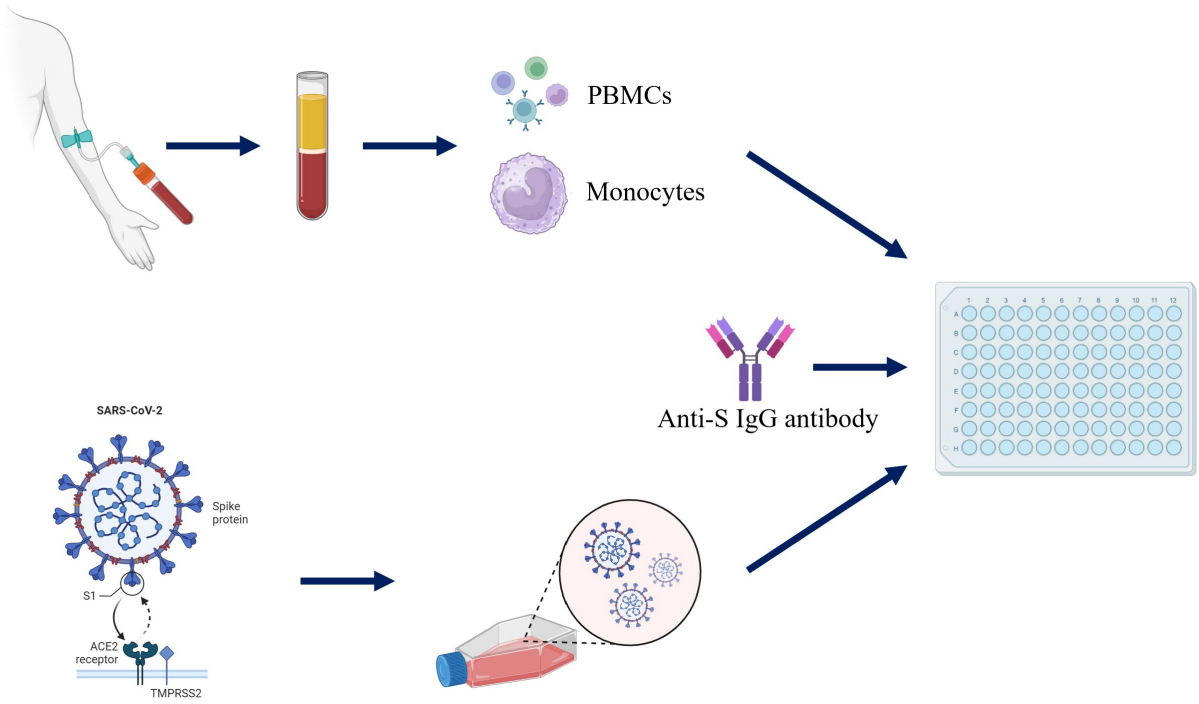

FIGURE 3: Antibody-dependent, cell-mediated virus inhibition (ADCVI) Assay. Blood is taken from four separate donors where effector cells (monocytes) are isolated and placed in 96-well plates with anti-S IgG antibody. Conditions measured included COVA1-18-only, COVA1-18 + monocytes, monocytes-only, virus-only, DEN3, and DEN3 + monocytes. All wells received infected VeroE6 cells and supernatant fluid was collected 72-hours later to measure viral RNA by quantitative RT-PCR.

CHAPTER 3.5: qPCR

Viral SARS-CoV-2 RNA, isolated from cell supernatant, was used as a template for the qPCRBIO SyGreen 1-step Go kit. Quantitative PCR was carried out with SARS-CoV-2 N-specific primers 5'-GGGGAACTTCTCCTGCTAGAAT-3' and 5'-CAGACATTTTGCTCTCAAGCTG-3'. SARS-CoV-2 copy numbers were quantified and

reported based on a standard curve generated by serial dilutions of a SARS-CoV-2 N plasmid standard.

CHAPTER 3.6: Analysis

The percentage of viral load decrease was calculated from qPCR values using the equation: 100-(RNA copy # obtained with effector cells and antibody/RNA copy # with controls) x100. Average copy numbers and standard deviations (SD) were calculated across all donors. The viral RNA copy numbers were transformed into a logarithmic scale, and a normality test was run, which determined the data was parametric. Paired T-tests were performed, and P-values <0.05 were deemed statistically significant. There was no adjustment made for multiple comparisons. All calculations and analyses were performed using Excel and Prism. All figures were created using BioRender.

CHAPTER 4: RESULTS

CHAPTER 4.1: Neutralization Assay

We first determined the neutralizing activity of COVA1-18 at various concentrations (FIGURE 4). Based on the neutralization curve, the IC50 was calculated at 0.32ug/mL. The IC90 value was calculated to be 4.95ug/mL. Based on these values, further experiments utilized COVA1-18 at 10ug/mL, which neutralized 96.3% of the extracellular virus.

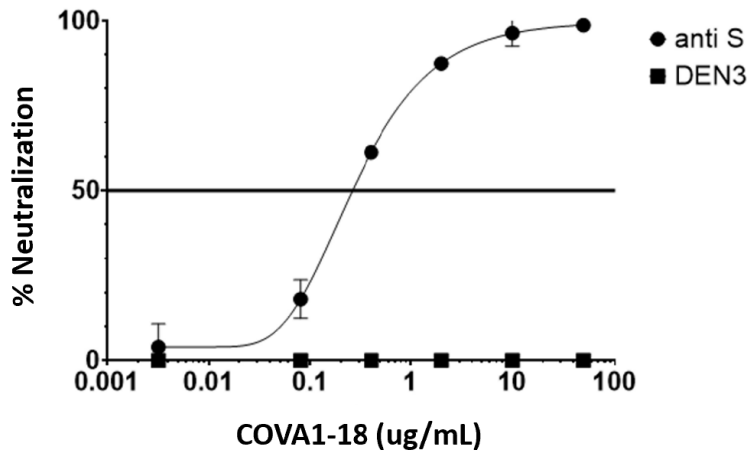

FIGURE 4: COVA1-18 neutralizes cell-free SARS-CoV-2. The horizontal line represents 50% neutralization.

CHAPTER 4.2: Blood Donor Characteristics

Blood was obtained for monocyte isolation from four donors consisting of three women and one man, with only one individual identifying as Asian/non-Hispanic. All other donors were classified as white/non-Hispanic. Donors' ages ranged from 25 to 35, and none reported any medications impacting immune cells or immune function. (**TABLE 1**). No additional information about the donors was provided.

Demographics	Donor 1	Donor 2	Donor 3	Donor 4
Sex	F	M	F	F
Age	33	35	25	28
Race/Ethnicity	Asian/Non-Hispanic	White/Non-Hispanic	White/Non-Hispanic	White/Non-Hispanic
Medications	None	None	Yes*	None

*Medications taken by Donor 3 were listed and were not found to have any impact on immune cells or immune function.

TABLE 1: Donor Information

CHAPTER 4.3: ADCVI Assay

To determine the effect of antibodies and monocytes on viral yield, antibody alone, antibody+ monocytes, and the indicated control conditions were incubated with infected VeroE6 cells. Overall, the results showed a significant reduction in viral load with antibody alone compared to controls (P=0.027), but there was greater reduction when antibody and monocytes were combined **(FIGURE 5)**

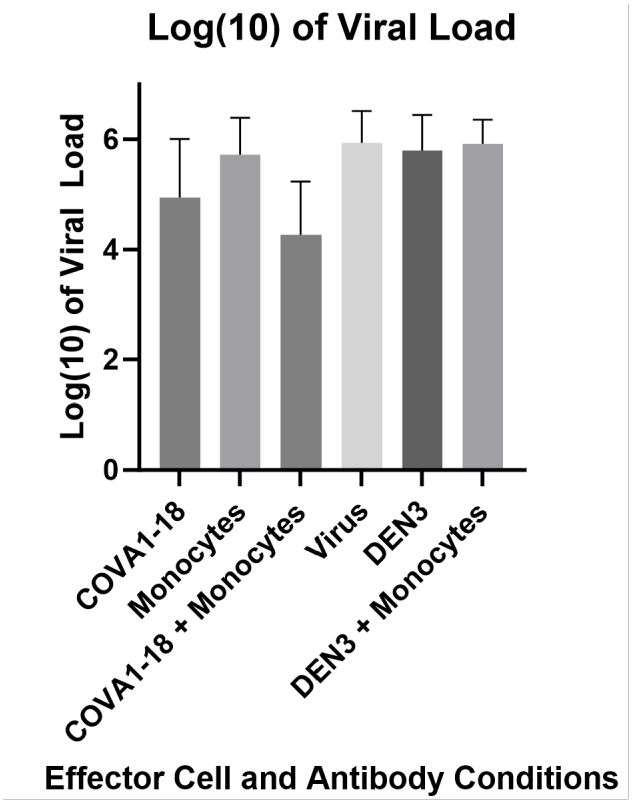

FIGURE 5: COVA1-18 combined with monocytes lowers viral yield from infected cells. Error bars represent standard deviations.

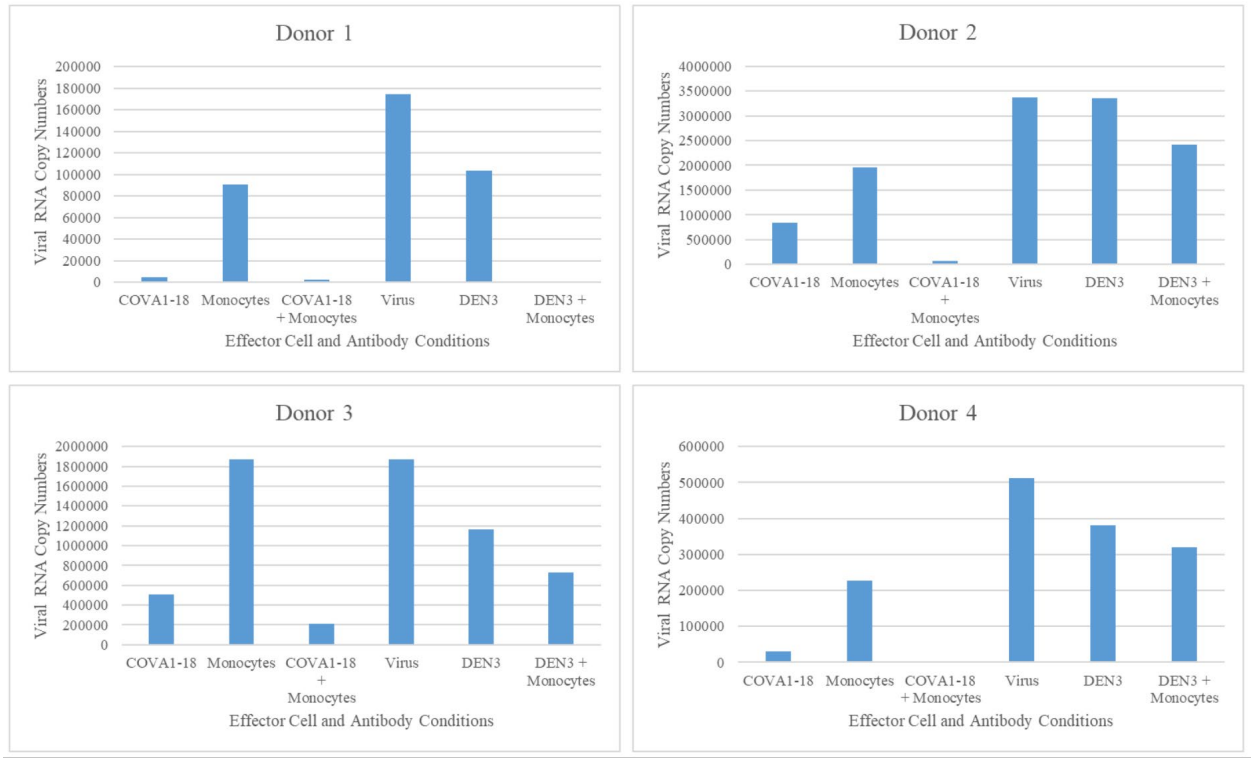

FIGURE 6: ADCVI assay results vary between donors. Virus yield is shown for each of the four monocyte donors under the conditions tested.

Variability in viral yield for each condition was noted between individual monocyte donors

(FIGURE 6).

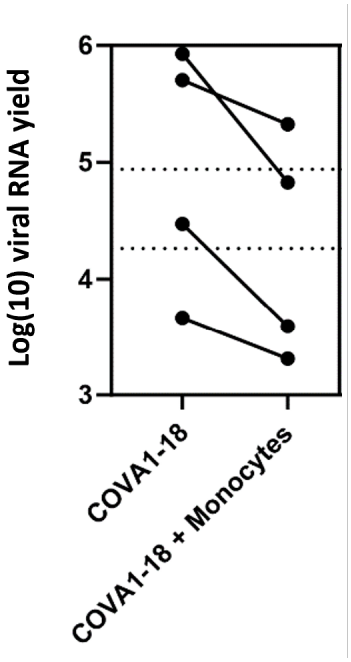

FIGURE 7: Combining mAb and monocytes resulted in reduced viral yield for each of the four donors.

On average, virus yield was 0.67 logs lower with COVA1-18 + monocytes compared to COVA1-18 alone (p=0.01, T-test on paired samples**; FIGURE 7, TABLE 2**). Compared to monocytes alone, COVA1-18 + monocytes reduced viral yield by 1.4 logs (p=0.004). Finally, COVA1-18+ monocytes reduced viral yield by 1.6 logs compared to the virus alone (i.e., with no mAb or monocytes; p=0.007).

Paired T-tests	P-value	R-squared	Mean of Differences	SD of Differences
Anti-S mAB + Monocytes vs. Anti-S mAB	0.03	0.81	-0.68	0.37
Anti-S mAB + Monocytes vs. Monocytes	0.004	0.96	-1.46	0.36
Anti-S mAB + Monocytes vs. Virus	0.0073	0.93	1.67	0.51

TABLE 2: Statistical analysis of ADCVI assay results. P-values are not corrected for multiple comparisons.

CHAPTER 5: CONCLUSION

We found that the combination of mAb and monocytes significantly reduced viral yield from infected cells when compared to mAb alone, to monocytes alone, or when neither mAb nor monocytes were present. The reduction of .67 logs when mAb+ monocytes and mAb alone are compared may be modest, but is consistent with the requirement for effector cell activity to impact viral yield from infected cells.

Few studies have focused on primary monocytes and how they interact with mAB in the presence of SARS-CoV-2. The ADCVI assay measures the effect of antibody and effector cell functions in reducing viral yield. Since combining antibody and monocytes results in more significant reductions in viral yield than antibodies or monocytes alone, enhancing interactions between antibody and monocytes could help improve the efficacy of monoclonal antibody therapies. Increasing interactions can be accomplished by mutating the Fc region of the antibody to increase engagement between Fc and FcγRs. Thus, using antibodies with mutations in the Fc fragment that increase binding to Fc receptors may further increase mAb efficacy in the clinical setting.

Although we showed a substantial decrease in the viral yield of infected cells in the presence of antibodies and monocytes, SARS-CoV-2 can infect primary monocytes. One study demonstrated that about ten percent of blood monocytes can become infected by SARS-CoV-2 via the FcγRs **(43)**. However, once infected, the monocytes undergo pyroptosis and do not replicate the virus **(43)**.

Limitations of this study included only testing one SARS-CoV-2 variant (Washington Strain), one neutralizing mAB (COVA1-18), and no polyclonal antibodies. Additional neutralizing and non-neutralizing antibodies should be evaluated using other virus strains to provide a more comprehensive analysis. Another limitation of this study is the target cell type chosen. VeroE6 cells, which are of non-human primate origin, were used, whereas other cells of human origin, including HEK293TACE2 and Calu3 cell types may be more relevant. However, Vero E6 cells are frequently used for most SARS-CoV-2 assays and proved to be adequate for our study. Finally, we used a small number of monocyte donors in the ADCVI assay. Pilot studies of this nature often begin with few donors to determine the effect size **(44,45)**. However, monocyte function is expected to vary between individuals and additional donors should be tested to confirm our findings**.** However, we expect the SD will likely not increase substantially, based on the slight variation seen between the SD's of the four donors tested.

It is of interest to determine the precise mechanisms that underlie the decrease in viral yield between COVA1-18 and COVA1-18+monocytes. We predict that ADCC, ADCP, the production of soluble substances (cytokines and chemokines), or all three are important mechanisms. Additionally, in-vivo experiments would determine if the effect size shown in this study scales to more complex conditions. Future studies will also test multiple SARS-CoV-2 variants, multiple mAbs, antibodies in blood produced by COVID-19 patients with varying disease severity, and multiple effector cell types, including but not limited to neutrophils and NK cells. Using multiple target cell types, including HEK293TACE2 and Calu3 cells would also be of interest.

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