ADDENDUM MATERIAL
DATE 8/10/21 ITEM NO. A004

Danielle Greene

From: Michael Morris [redacted]
Sent: Wednesday, August 11, 2021 8:27 AM
To: COB_mail
Subject: URGENT;STOP THE MANDATE

Our nurses fought for us all. They went to work everyday and helped people recover. They held the hands of our loved ones when we weren't allowed to. They are human and deserve to be treated as such. Allow them to choose for themselves. NO MANDATES.

Sent from my iPhone

Danielle Greene

From: Paul LaValley <[redacted]
Sent: Wednesday, August 11, 2021 8:16 AM
To: Dist1@pima.gov; DIST2; District3; Dist4@pima.gov; Dist5@pima.gov; Chuck Huckelberry; COB_mail; Mable; Garret@790knst.com; KELLY@kellyjohnwalker.com
Subject: Urgent! Stop The Mandates

[redacted]

Dear Sirs,
I, like many, had the virus, thus I don't require the added protection of a vaccination with a 75% effective rate, for a virus that has a 99.8% recovery rate...the recovery rate is higher than that if one uses the banned Therapeutics or Prophalactics' such as HCQ/Ivermectin.

It's LUNACY to even consider mandating this or any experimental drug with a growing history of adverse reactions, and death. especially since the borders are wide open...no mention of fixing that from you with a blanket travel ban. I don't care what loophole your lawyers may find in the law...it's wrong!

Why no mention of the deaths from Cigarette smoking, or traffic accidents? they kill more than the virus.

Do the right thing. Don't be a part of the biggest hoax ever put upon the American people.
Groups are forming as I speak. If you vote for this travesty of liberty, you will be protested, and exposed as the enemy of the people, which is what you will be if you vote for Mandatory Vaccinations aka: tyranny.

Sincerely,
Paul LaValley- Green Valley

Danielle Greene

From: Heather <[redacted]
Sent: Wednesday, August 11, 2021 7:34 AM
To: COB_mail
Subject: Urgent: stop the mandates

To whom it may concern;

It is imperative you work for who voted you in and stop all Covid related mandates. To include mandated mask wearing and mandated cv vaccines. It absolutely should be up to the individual to make the choice based on individual circumstances and individual health status. There are absolutely no medical term or long term studies and data regarding the safety and efficacy of this particular vaccine. There are valid medical, philosophical, and religious reasons that people cannot take this vaccine. To ignore that Would be irresponsible and harmful.

Heather

Danielle Greene

From: [redacted]
Sent: Wednesday, August 11, 2021 6:56 AM
To: COB_mail
Subject: URGENT STOP THE MANDATES

I am writing to express my support to stop the mandates. The REAL science tells us something totally different from what Dr Fauci is saying. The websites of the CDC and FDA have solid studies that were done to show the real science that they are ignoring.
Under our Constitution we have freedoms and rights and no one should have their job put on the line for refusing an experimental treatment...especially for a virus that has a 97-99% recovery rate! Our healthcare workers stood up to the challenges in the past year and are capable of deciding what is best for their bodies,..not the government or their company.
Thank you.
Kathy Hilgendorf

Sent from my iPad

Danielle Greene

From: liliy wentz <[redacted]
Sent: Tuesday, August 10, 2021 10:03 PM
To: COB_mail
Subject: URGENT; STOP THE MANDATE

[redacted]

We want to thank you for taking the time to read this email.

We the people and health care workers will stand together to flight the Covid-19 vaccine mandated. We as health care workers and community members are willing to do whatever it takes to stop the violation on our civil rights as Americans. We have had our first amendment right violated. We are NOT giving up our individual freedoms for any reason. This needs to stop! This vaccine will not stop Covid-19. By mandating the Covid -19 vaccine you are forcing us to put the following poison into our bodies:

- Graphene Oxide
- Alphanumeric codes

The mainstream media is not being truthful, Big tech and social media have been blocking any real information. For this I urge you to do your own research. Read the 193 page Covid -19 vaccine patent and tell me that you are 100% sure this will not have any short terms or long term affect on our bodies, can you do that?

Kind regards,

Lily Angulo

Danielle Greene

From: Kevin Cochran <[redacted]>
Sent: Tuesday, August 10, 2021 9:59 PM
To: COB_mail
Subject: URGENT;STOP THE MANDATE!

[redacted]

To the pima county board of supervisors

I'm writing to you today to ask that you vote to stop this mandate on medical workers in pima County I am an imaging technologist who has worked my entire career helping the people of pima county in their darkest hours working level 1 trauma and I have worked week after week during the entirety of this pandemic with not enough ppe and willingly putting myself in harms way I understood that this was the field of work I chose so that I might be able to help people in need and I do that selflessly every day only to now be told that if I do not take this experimental vaccine (and that is definitely what this is, we have no real long term data on the risks of this treatment) that if I don't blindly take this I will be terminated from my employment I will not be able to provide for my family it's wrong it is immoral and for those reason I beg that you would have compassion for the hard work we do and and our concern for the rights that we hold so dear in this country! And vote no on these vaccine mandates!

Sincerely
Kevin A Cochran RT(R, CT, ARRT)

Danielle Greene

From: Shauna Tippetts <[redacted]>
Sent: Tuesday, August 10, 2021 7:15 PM
To: COB_mail
Subject: STOP THE MANDATE!!!!!'

[redacted]

I am demanding you to stop the DEATH SHOT mandate! You are evil and I beg God to avenge his people --
Sent from Gmail Mobile

Jessica Kopfmann

From: Michael Morris [redacted]
Sent: Wednesday, August 11, 2021 8:51 AM
To: COB_mail
Subject: URGENT;STOP THE MANDATE

Natural immunity is not being excepted as a reason to opt out of the forced vaccination. That is simply unscientific. Ivermectin is not being excepted as a preventative or being used as an early treatment. That is unscientific as well. I implore you all to look into things for yourselves. Go to FLCCC.net and hear from doctors across the world. STOP THE MANDATES!!!!!

Sent from my iPhone

Jessica Kopfmann

From: Monica Mott <[redacted]
Sent: Wednesday, August 11, 2021 8:45 AM
To: COB_mail
Subject: URGENT

STOP THE MANDATE!

Sent via the Samsung Galaxy Note9, an AT&T 5G Evolution capable smartphone
Get Outlook for Android

Danielle Greene

From: Marcy Heiman <[redacted]
Sent: Wednesday, August 11, 2021 7:06 AM
To: COB_mail
Subject: Stop all COvid mandates

Please stop all Covid mandates. They are not necessary and are all unconstitutional.

Thank you.

Marcy
Sent from my iPhone

Danielle Greene

From: Chris Heiman <[redacted]
Sent: Wednesday, August 11, 2021 7:01 AM
To: COB_mail
Subject: Urgent STOP the Mandates

Please vote NO on all mandates. This is harmful to everyone!
Thank you

Chris Heiman

Jessica Kopfmann

From: Jessica Luna <[redacted]
Sent: Wednesday, August 11, 2021 8:37 AM
To: COB_mail
Subject: URGENT. STOP THE MANDATES

Hello, I'm writing you today to ask you to please stop the mandate for covid shots for healthcare workers. As an RN myself, I have seen adverse reactions and I believe in freedom of choice. Anytime there is risk with any medical procedure, there must be choice. I urge you to please vote against this medical mandate, and allow our healthcare workers to make a choice for themselves. Thank you.

Jessica Luna, RN, BSN

Jessica Kopfmann

From: michelle boyd <[redacted]
Sent: Wednesday, August 11, 2021 8:54 AM
To: COB_mail
Subject: URGENT; STOP THE MANDATE!

As a healthcare employee, I strongly disagree with the vaccine mandate. Healthcare choices should be the decision of the people. Healthcare workers are in the business to care for people and have and always will take proper precautions. COVID 19 is not the only virus that risks patients and workers alike. The precautions are followed and are no more risk than any other medical condition. This segregation of healthcare and other persons is unfair and unjust. To blame solely this Virus on unvaccinated people is not only a lie but also unconstitutional and violates all civil rights. My body.. My Choice!

Sent from my iPhone

Jessica Kopfmann

From: Kip Congdon <[redacted]
Sent: Tuesday, August 10, 2021 6:59 AM
To: COB_mail
Subject: Request this email be read into the record for the Pima County Board of Supervisors meeting 8/10/21

Dear Sir or Madame:

Regarding Mandatory Vaccination for all health care workers in Pima County. The COVID 19 vaccines currently available have not been approved by the FDA and are for emergency use only.. This vaccine has not completed the trial protocols that every other vaccine in U.S.
history have followed. In fact, in the United States, so far there have been over 12,000 deaths associated with these four "medications." The inventor of mRNA technology, Dr. Robert Malone has publicly stated that the public health leadership has "stepped over the line and is now violating the bedrock principles which form the foundation upon which the ethics of clinical research are built." (https://www.totalhealth.co.uk/blog/are-people-getting-full-facts-covid-vaccine-risks
). When animal trials were conducted with vaccines developed for the SARS (severe acute respiratory syndrome) and MERS (middle eastern respiratory syndrome) virus, ferrets were among the animals used. After the initial vaccine injection the animals appeared to do well. However after being re-exposed to the virus, the animal immune system recognized the spike protein in the vaccine, which had entered every organ in the animal and attacked it, killing the ferret. This response is called antibody dependent enhancement. Humans are the animals used in the COVID 19 animal trials. If we respond to virus re-exposure in the fall as the ferrets did, you will see many very ill people, and probably many deaths. If this happens and you have mandated that health workers get the "Vaccine" you have the potential to be held criminally liable, given that you were informed of the inherent risks.

With respect to the K-12 Mask Mandate, from a scientific standpoint, masks simply do not work. The diameter of the pores in either paper or cloth masks range from 80,000 nanometers (nm) to 500,000 nm. The diameter of the COVID molecule is between 40 and 160 nm. The virus easily passes through the mask pores. From a physical standpoint it is not healthy for kids to be breathing CO_2 for 8 to 10 hours a day. If people feel compelled to wear a mask that is their right. But it isn't your right to tell all students they must wear a mask when it doesn't make any sense to do so.

Please consider the point I've made above. Thank you.

--
Kip Congdon
[redacted]

Jessica Kopfmann

From: Jill Kimmerle [redacted]
Sent: Wednesday, August 11, 2021 9:04 AM
To: COB_mail
Subject: No Mandates

NO mandates for Health Care Workers and employees! Freedom of choice over our bodies!
Jillianne Kimmerle

Sent from my iPhone

Jessica Kopfmann

From: Tonda Franklin <[redacted]
Sent: Wednesday, August 11, 2021 9:18 AM
To: COB_mail
Subject: Mandatory Covid Vax

To whom this may concern,
Myself and many others employee you to halt the mandate for Covid shot for out county. Our rights are being stolen. I am a nurse and there are thousands like me that will refuse and walk out. There is already a nursing shortage and those that continue to support our community feel betrayed for all we did for our employers and our community with such a lack of resources.
I am praying that you all make the right choice to protect our constitutional rights so our community doesn't suffer a lack of healthcare and resources I also implore you to bring to light all the prevention and early treatment that has much research and has saved millions of lives. Give our physicians/provider their voice who are not suppressing but validating data on Ivermectin, HCQ, high dose VitaminC and Vitamin D. The censorship that has been allowed by our government organizations and our news and social media is communist. Please stand up for what is right. Stand up for our constitution!
Thank you

Sent from my iPhone

Jessica Kopfmann

From: Tonda Franklin [redacted]
Sent: Wednesday, August 11, 2021 9:22 AM
To: COB_mail
Subject: Urgent! Stop the mandates!

>
> To whom this may concern,
> Myself and many others employee you to halt the mandate for Covid shot for out county. Our rights are being stolen. I am a nurse and there are thousands like me that will refuse and walk out. There is already a nursing shortage and those that continue to support our community feel betrayed for all we did for our employers and our community with such a lack of resources.
> I am praying that you all make the right choice to protect our
> constitutional rights so our community doesn't suffer a lack of healthcare and resources I also implore you to bring to light all the prevention and early treatment that has much research and has saved millions of lives. Give our physicians/provider their voice who are not suppressing but validating data on Ivermectin, HCQ, high dose VitaminC and Vitamin D. The censorship that has been allowed by our government organizations and our news and social media is communist. Please stand up for what is right. Stand up for our constitution!
> Thank you
>
>

Jessica Kopfmann

From:	Peter LaJoy <[redacted]
Sent:	Wednesday, August 11, 2021 9:18 AM
To:	COB_mail
Cc:	'peterlajoypt@gmail.com'; peteramccullough@gmail.com
Subject:	URGENT STOP THE MANDATE Look at this
Attachments:	CIRCRESAHA.121.318902.pdf; httpswww.nature.comarticless41564-020-00789-5.pdf.pdf; Immunity COVID study.pdf; Clinical outcomes after early ambulatory multidrug therapy for high-risk....pdf; pitfalls.pdf; Ivermectin study.pdf; 1-s2.0-S0002934320306732-main.pdf; Fact Sheet COVID VAX May 2021.docx

Greetings,
Here are the facts
There are treatments available that work even prevention. Studies included.
The Spike is the agent of COVID -19 now we know as affecting the cardiovascular system. The current use of vaccination uses the spike . This has been done before. See studies included.

Mandates for a corona virus vaccine are counterproductive to the health of AZ.

Sincerely,

Peter LaJoy PT BS PT DPT

Peter LaJoy | PT

Physical Therapy
Copper Queen Community Hospital
10524 E. Highway 92, Palominas, Arizona, 85615
Phone Number: [redacted]
Email: plajoy@cqch.org
www.cqch.org

Circulation Research

RESEARCH LETTER

SARS-CoV-2 Spike Protein Impairs Endothelial Function via Downregulation of ACE 2

Yuyang Lei,* Jiao Zhang,* Cara R. Schiavon, Ming He, Lili Chen, Hui Shen, Yichi Zhang, Qian Yin, Yoshitake Cho, Leonardo Andrade, Gerald S. Shadel, Mark Hepokoski, Ting Lei, Hongliang Wang, Jin Zhang, Jason X.-J. Yuan, Atul Malhotra, Uri Manor,† Shengpeng Wang,† Zu-Yi Yuan,† John Y-J. Shyy†

SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) infection relies on the binding of S protein (Spike glycoprotein) to ACE (angiotensin-converting enzyme) 2 in the host cells. Vascular endothelium can be infected by SARS-CoV-2,[1] which triggers mitochondrial reactive oxygen species production and glycolytic shift.[2] Paradoxically, ACE2 is protective in the cardiovascular system, and SARS-CoV-1 S protein promotes lung injury by decreasing the level of ACE2 in the infected lungs.[3] In the current study, we show that S protein alone can damage vascular endothelial cells (ECs) by downregulating ACE2 and consequently inhibiting mitochondrial function.

We administered a pseudovirus expressing S protein (Pseu-Spike) to Syrian hamsters intratracheally. Lung damage was apparent in animals receiving Pseu-Spike, revealed by thickening of the alveolar septa and increased infiltration of mononuclear cells (Figure [A]). AMPK (AMP-activated protein kinase) phosphorylates ACE2 Ser-680, MDM2 (murine double minute 2) ubiquitinates ACE2 Lys-788, and crosstalk between AMPK and MDM2 determines the ACE2 level.[4] In the damaged lungs, levels of pAMPK (phospho-AMPK), pACE2 (phospho-ACE2), and ACE2 decreased but those of MDM2 increased (Figure [B], i). Furthermore, complementary increased and decreased phosphorylation of eNOS (endothelial NO synthase) Thr-494 and Ser-1176 indicated impaired eNOS activity. These changes of pACE2, ACE2, MDM2 expression, and AMPK activity in endothelium were recapitulated by in vitro experiments using pulmonary arterial ECs infected with Pseu-Spike which was rescued by treatment with N-acetyl-L-cysteine, a reactive oxygen species inhibitor (Figure [B], ii).

We next studied the impact of S protein on mitochondrial function. Confocal images of ECs treated with S1 protein revealed increased mitochondrial fragmentation, indicating altered mitochondrial dynamics (Figure [C], i). To examine whether these mitochondrial changes were due, in part, to the decreased amount of ACE2, we overexpressed ACE2 S680D (ACE2-D, a phospho-mimetic ACE2 with increased stability) or S680L (ACE2-L, a dephospho-mimetic with decreased stability)[4] in ECs. As shown in Figure [C], ii, ECs with ACE2-L had a higher number of fragmented mitochondria when compared to those with ACE2-D. Performing oxygen consumption rate and extracellular acidification rate assays, we found that ECs overexpressing ACE2-L had reduced basal mitochondrial respiration, ATP production, and maximal respiration compared to ECs overexpressing ACE2-D (Figure [D], i). Moreover, ACE2-L overexpression caused increased basal acidification rate, glucose-induced glycolysis, maximal glycolytic capacity, and glycolytic reserve (Figure [D], ii). Also, ECs incubated with S1 protein had attenuated mitochondrial function but increased

Key Words: angiotensin-converting enzyme 2 ■ endothelium ■ SARS-CoV-2

Meet the First Author, see p 1239

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*Y. Lei and J. Zhang contributed equally.

†U. Manor, S. Wang, Z.-Y. Yuan, and J.Y.-J. Shyy contributed equally as senior authors.

For Sources of Funding and Disclosures, see page 1324.

Circulation Research is available at www.ahajournals.org/journal/res

Nonstandard Abbreviation and Acronyms

ACE	angiotensin-converting enzyme
ECs	endothelial cells
eNOS	endothelial NO synthase
pACE2	phospho-ACE2
pAMPK	phospho-AMPK
S protein	Spike glycoprotein

glycolysis, when compared with control cells treated with IgG (Figure [D], iii and iv). We also compared the expressions of mitochondria- and glycolysis-related genes in lung ECs isolated from ACE2-D or ACE2-L knock-in mice.[4] Shown in Figure [E], the mRNA levels of *NRF1*, *HO1*, and *TFAM* (mitochondria biogenesis-related genes) were increased, whereas those of *HK2*, *PFKFB3*, and *ENO2* (glycolysis-related genes) were decreased in lung ECs in ACE2-D mice, as compared to those in ACE2-L mice.

SARS-CoV-2 infection induces EC inflammation, leading to endotheliitis.[1,5] Because S protein decreased ACE2 level and impaired NO bioavailability, we examined whether S protein entry is indispensable for dysfunctional endothelium. As shown in Figure [F], i, the endothelium-dependent vasodilation induced by acetylcholine was impaired in pulmonary arteries isolated from Pseu-Spike-administered hamsters, whereas the endothelium-independent vasodilation induced by sodium nitroprusside was not affected. We also compared the acetylcholine- and sodium nitroprusside–induced vasodilation of pulmonary vessels from ACE2-D or ACE2-L mice. As anticipated, acetylcholine-induced vasodilation was hindered in pulmonary arteries isolated from ACE2-L mice in comparison to ACE2-D mice (Figure [F], ii). There was, however, little difference in sodium nitroprusside–induced vasodilation between ACE2-D and ACE-L animals.

Although the use of a noninfectious pseudovirus is a limitation to this study, our data reveals that S protein alone can damage endothelium, manifested by impaired mitochondrial function and eNOS activity but increased glycolysis. It appears that S protein in ECs increases redox stress which may lead to AMPK deactivation, MDM2 upregulation, and ultimately ACE2 destabilization.[4] Although these findings need to be confirmed with the SARS-CoV-2 virus in the future study, it seems paradoxical that ACE2 reduction by S protein would decrease the virus infectivity, thereby protecting endothelium. However, a dysregulated renin-angiotensin system due to ACE2 reduction may exacerbate endothelial dysfunction, leading to endotheliitis. Collectively, our results suggest that the S protein-exerted EC damage overrides the decreased virus infectivity. This conclusion suggests that vaccination-generated antibody and/or exogenous antibody against S protein not only protects the host from SARS-CoV-2 infectivity but also inhibits S protein-imposed endothelial injury.

ARTICLE INFORMATION

Data Availability

The data that support the findings of this study, including statistical analyses and reagents used, are available from the corresponding author upon request.

Affiliations

Cardiology, First Affiliated Hospital of Xi'an Jiaotong University (Y.L., Jiao Zhang, Z.-Y.Y.). Cardiovascular Research Center, School of Basic Medical Sciences (Y.L., Jiao Zhang, L.C., Q.Y., S.W.). Pathology, School of Basic Medical Sciences (T.L.), Pathogen Biology and Immunology, School of Basic Medical Sciences (H.W.), Xi'an Jiaotong University Health Science Center. Cardiology, Department of Medicine (Jiao Zhang, M. He, H.S., Y.Z., Y.C., J.Y.-J.S.), Pulmonary, Critical Care and Sleep Medicine, Department of Medicine (M. Hepokoski, J.X.-J.Y., A.M.), and Pharmacology (Jin Zhang), University of California, San Diego, La Jolla, CA. Waitt Advanced Biophotonics Center (C.R.S., L.A., U.M.). Molecular and Cellular Biology Laboratory, Salk Institute for Biological Studies, La Jolla, CA (C.R.S., G.S.S.). Cardiology, the Affiliated Hospital of Yangzhou University (H.S.).

Sources of Funding

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Disclosures

None.

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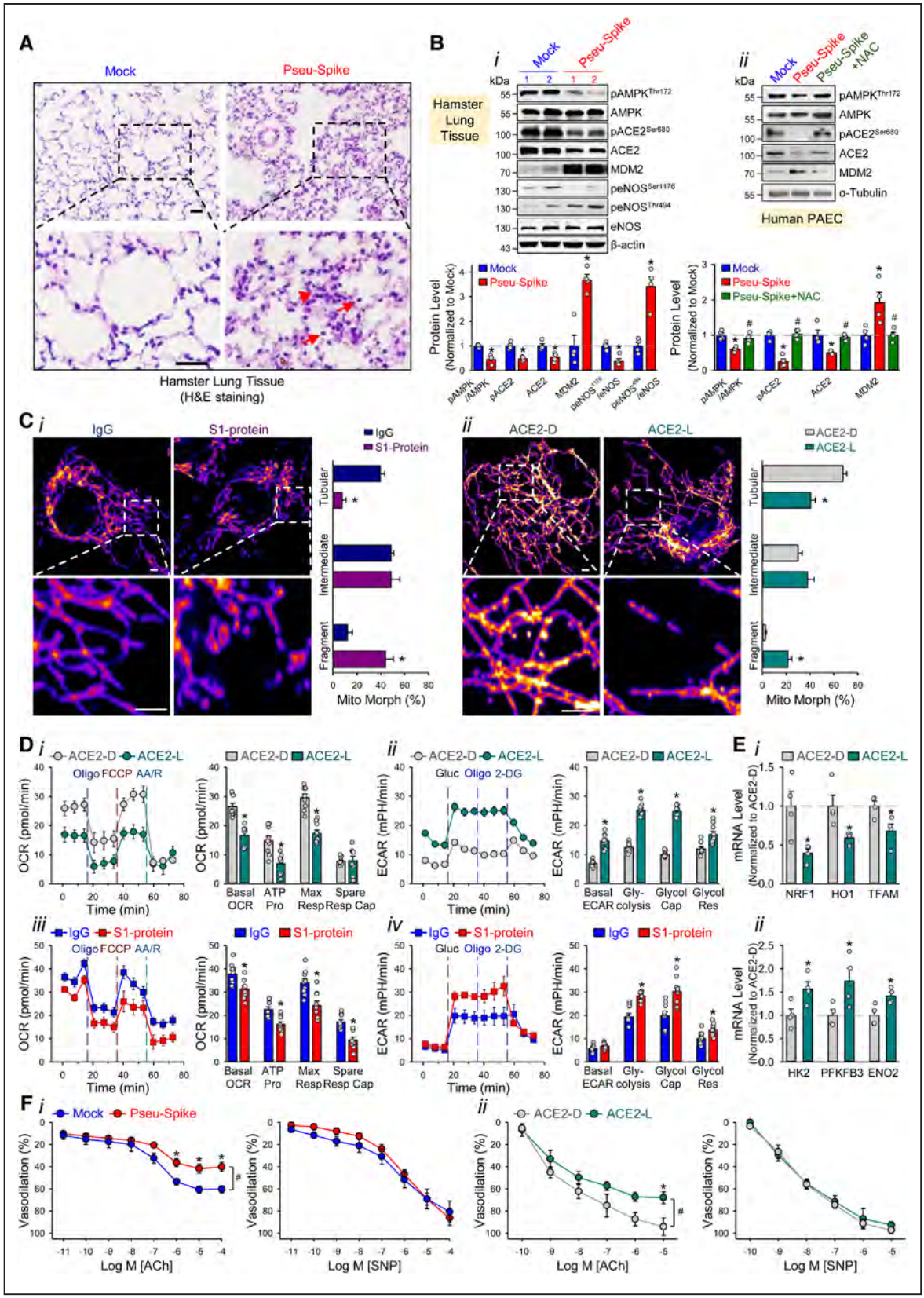

Figure. SARS-CoV-2 (Severe acute respiratory syndrome coronavirus 2) Spike protein exacerbates endothelial cell (EC) function via ACE (angiotensin-converting enzyme) 2 downregulation and mitochondrial impairment.

A, Representative H&E histopathology of lung specimens from 8- to 12 wk-old male Syrian hamsters 5-day post administration of pseudovirus overexpressing Spike protein (Pseu-Spike) or mock virus in control group (n=3 mice per group, 1×10^8 PFU). Thickened alveolar septa (red arrowhead) and mononuclear cell (red arrow). Scale bar=20 μm. **B,** Pseu-Spike (n=4) or mock virus (n=4)–infected hamster (*Continued*)

Figure Continued. lungs were subjected to Western blot analysis for pAMPK (phospho-AMPK) T172, AMPK, pACE2 (phospho angiotensin-converting enzyme) S680, ACE 2, MDM2, peNOS S1176, peNOS T494, eNOS (endothelial NO synthase), and β-actin (**B**, i). Human pulmonary arterial EC (PAECs) were infected with Pseu-Spike or mock virus for 24 h with or without N-acetyl-L-cysteine (NAC; 5 mmol/L) pretreatment for 2 h. The protein extracts were analyzed by Western blot using antibodies against proteins as indicated (n=4; **B**, ii). **C,** Representative confocal images of mitochondrial morphology of ECs treated with human recombinant S1 protein or IgG (4 μg/mL) for 24 h (**C**, i) or infected with human adenovirus ACE2 S680D (ACE2-D) or ACE2 S680L (ACE2-L; 10 MOI) for 48 h (**C**, ii). Mitochondria were visualized using TOM20 antibody (n=4, 50 cells counted for each replicate). Scale bar=2.5 μm. Tubular: the majority of mitochondria in ECs was >10 μm in length; Intermediate: the mitochondria were <≈10 μm; Fragment: the majority of mitochondria were spherical (no clear length or width). **D**, Measurement of oxygen consumption rate (OCR, **D**, i and iii) and extracellular acidification rate (ECAR, **D**, ii and iv) in ECs infected with ACE2-D vs ACE2-L (10 MOI) for 48 h (n=3) or treated with IgG vs S1 protein (4 μg/mL) for 24 h (n=3). **E**, Real-time quantitative polymerase chain reaction analysis of the indicated mRNA levels in lung ECs from ACE2-D (n=4) and ACE2-L (n=4) knock-in mice. Eight-week-old ACE2-D and ACE2-L male mice with C57BL/6 background were used. **F**, Dose-response curves of acetylcholine (ACh, **left**)- and sodium nitroprusside (SNP, **right**)–mediated relaxation on the tension of phenylephrine (1 μmol/L) precontracted intrapulmonary artery stripes from Pseu-Spike-(ACh n=8, SNP n=5) or mock (ACh n=6, SNP n=5) virus–infected Syrian hamsters (1×10^8 PFU; **F**, i) and ACE2-D (n=6) or ACE2-L (n=5) mice (**F**, ii). The animal experiments were approved by the ethical committee of Xi'an Jiaotong University. 2-DG indicates 2-Deoxy-D-glucose; ACE2-D, a phospho-mimetic ACE2 with increased stability; ACE2-L, a dephospho-mimetic ACE2 with decreased stability; AMPK, AMP-activated protein kinase; AA/R, antimycin A&Rotenone; ENO2, enolase 2; FCCP, carbonyl cyanide-p-(trifluoromethoxy)phenylhydrazone; H&E, Hematoxylin and Eosin; HK2, hexokinase 2; HO1, heme oxygenase-1; MDM2, murine double minute 2; MOI, multiplicity of infection; NRF1, nuclear respiratory factor 1; peNOS, phospho-eNOS; PFKFB3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3; Resp, respiration; and TFAM, transcription factor A, mitochondrial.

nature microbiology

PERSPECTIVE

https://doi.org/10.1038/s41564-020-00789-5

Check for updates

Antibody-dependent enhancement and SARS-CoV-2 vaccines and therapies

Wen Shi Lee[1], Adam K. Wheatley[1,2], Stephen J. Kent[1,2,3] ✉ and Brandon J. DeKosky[4,5,6] ✉

Antibody-based drugs and vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are being expedited through preclinical and clinical development. Data from the study of SARS-CoV and other respiratory viruses suggest that anti-SARS-CoV-2 antibodies could exacerbate COVID-19 through antibody-dependent enhancement (ADE). Previous respiratory syncytial virus and dengue virus vaccine studies revealed human clinical safety risks related to ADE, resulting in failed vaccine trials. Here, we describe key ADE mechanisms and discuss mitigation strategies for SARS-CoV-2 vaccines and therapies in development. We also outline recently published data to evaluate the risks and opportunities for antibody-based protection against SARS-CoV-2.

The emergence and rapid global spread of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19), has resulted in substantial global morbidity and mortality along with widespread social and economic disruption. SARS-CoV-2 is a betacoronavirus closely related to SARS-CoV (with ~80% sequence identity), which caused the SARS outbreak in 2002. Its next closest human coronavirus relative is Middle East respiratory syndrome-related coronavirus (MERS-CoV; ~54% sequence identity), which caused Middle East respiratory syndrome in 2012 (refs. [1,2]). SARS-CoV-2 is also genetically related to other endemic human coronaviruses that cause milder infections: HCoV-HKU1 (~52% sequence identity), HCoV-OC43 (~51%), HCoV-NL63 (~49%) and HCoV-229E (~48%)[1]. SARS-CoV-2 is even more closely related to coronaviruses identified in horseshoe bats, suggesting that horseshoe bats are the primary animal reservoir with a possible intermediate transmission event in pangolins[3].

Cellular entry of SARS-CoV-2 is mediated by the binding of the viral spike (S) protein to its cellular receptor, angiotensin-converting enzyme 2 (ACE2)[4,5]. Other host entry factors have been identified, including neuropilin-1 (refs. [6,7]) and TMPRSS2, a transmembrane serine protease involved in S protein maturation[4]. The SARS-CoV-2 S protein consists of the S1 subunit, which contains the receptor binding domain (RBD), and the S2 subunit, which mediates membrane fusion for viral entry[8]. A major goal of vaccine and therapeutic development is to generate antibodies that prevent the entry of SARS-CoV-2 into cells by blocking either ACE2–RBD binding interactions or S-mediated membrane fusion.

One potential hurdle for antibody-based vaccines and therapeutics is the risk of exacerbating COVID-19 severity via antibody-dependent enhancement (ADE). ADE can increase the severity of multiple viral infections, including other respiratory viruses such as respiratory syncytial virus (RSV)[9,10] and measles[11,12]. ADE in respiratory infections is included in a broader category named enhanced respiratory disease (ERD), which also includes non-antibody-based mechanisms such as cytokine cascades and cell-mediated immunopathology (Box 1). ADE caused by enhanced viral replication has been observed for other viruses that infect macrophages, including dengue virus[13,14] and feline infectious peritonitis virus (FIPV)[15]. Furthermore, ADE and ERD has been reported for SARS-CoV and MERS-CoV both in vitro and in vivo. The extent to which ADE contributes to COVID-19 immunopathology is being actively investigated.

In this Perspective, we discuss the possible mechanisms of ADE in SARS-CoV-2 and outline several risk mitigation principles for vaccines and therapeutics. We also highlight which types of studies are likely to reveal the relevance of ADE in COVID-19 disease pathology and examine how the emerging data might influence clinical interventions.

Mechanisms of ADE

ADE has been documented to occur through two distinct mechanisms in viral infections: by enhanced antibody-mediated virus uptake into Fc gamma receptor IIa (FcγRIIa)-expressing phagocytic cells leading to increased viral infection and replication, or by excessive antibody Fc-mediated effector functions or immune complex formation causing enhanced inflammation and immunopathology (Fig. 1, Box 1). Both ADE pathways can occur when non-neutralizing antibodies or antibodies at sub-neutralizing levels bind to viral antigens without blocking or clearing infection. ADE can be measured in several ways, including in vitro assays (which are most common for the first mechanism involving FcγRIIa-mediated enhancement of infection in phagocytes), immunopathology or lung pathology. ADE via FcγRIIa-mediated endocytosis into phagocytic cells can be observed in vitro and has been extensively studied for macrophage-tropic viruses, including dengue virus in humans[16] and FIPV in cats[15]. In this mechanism, non-neutralizing antibodies bind to the viral surface and traffic virions directly to macrophages, which then internalize the virions and become productively infected. Since many antibodies against different dengue serotypes

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Box 1 | ADE and ERD

ERD

ERD describes severe clinical presentations of respiratory viral infections associated with medical interventions (especially vaccines). Similar clinical presentations can occur as a result of natural infections, and so ERD is detected during preclinical and clinical trials by comparing the distribution of disease severities between the intervention and placebo study arms. ERD can be associated with a broad range of molecular mechanisms, including FcR-dependent antibody activity and complement activation (that is, ADE), but also to other antibody-independent mechanisms such as tissue cell death, cytokine release and/or local immune cell activation.

ADE

ADE can be broadly categorized into two different types based on the molecular mechanisms involved:

ADE via enhanced infection. Higher infection rates of target cells occur in an antibody-dependent manner mediated by Fc–FcR interactions. ADE via enhanced infection is commonly measured using in vitro assays detecting the antibody-dependent infection of cells expressing FcγRIIa, such as monocytes and macrophages. The link between in vitro ADE assay results and clinical relevance is often implied, rather than directly observed. Dengue virus represents the best documented example of clinical ADE via enhanced infection.

ADE via enhanced immune activation. Enhanced disease and immunopathology are caused by excessive Fc-mediated effector functions and immune complex formation in an antibody-dependent manner. The antibodies associated with enhanced disease are often non-neutralizing. ADE of this type is usually examined in vivo by detecting exacerbated disease symptoms, including immunopathology and inflammatory markers, and is most clearly associated with respiratory viral infections. RSV and measles are well-documented examples of ADE caused by enhanced immune activation.

ERD and ADE (of the second type described above) are often identified by clinical data, including symptom prevalence and disease severity, rather than by the specific molecular mechanisms that drive severe disease. The presence of complex feedback loops between different arms of the immune system makes it very difficult (although not impossible) to conclusively determine molecular mechanisms of ADE and ERD in human and animal studies, even if the clinical data supporting ADE and ERD are quite clear. Many different measurements and assays are used to track ADE and ERD, which can vary based on the specific virus, preclinical and/or clinical protocols, biological samples collected and in vitro techniques used.

Respiratory ADE is a specific subset of ERD.

are cross-reactive but non-neutralizing, secondary infections with heterologous strains can result in increased viral replication and more severe disease, leading to major safety risks as reported in a recent dengue vaccine trial[13,14]. In other vaccine studies, cats immunized against the FIPV S protein or passively infused with anti-FIPV antibodies had lower survival rates when challenged with FIPV compared to control groups[17]. Non-neutralizing antibodies, or antibodies at sub-neutralizing levels, enhanced entry into alveolar and peritoneal macrophages[18], which were thought to disseminate infection and worsen disease outcome[19].

In the second described ADE mechanism that is best exemplified by respiratory pathogens, Fc-mediated antibody effector functions can enhance respiratory disease by initiating a powerful immune cascade that results in observable lung pathology[20,21]. Fc-mediated activation of local and circulating innate immune cells such as monocytes, macrophages, neutrophils, dendritic cells and natural killer cells can lead to dysregulated immune activation despite their potential effectiveness at clearing virus-infected cells and debris. For non-macrophage tropic respiratory viruses such as RSV and measles, non-neutralizing antibodies have been shown to induce ADE and ERD by forming immune complexes that deposit into airway tissues and activate cytokine and complement pathways, resulting in inflammation, airway obstruction and, in severe cases, leading to acute respiratory distress syndrome[10,11,22,23]. These prior observations of ADE with RSV and measles have many similarities to known COVID-19 clinical presentations. For example, over-activation of the complement cascade has been shown to contribute to inflammatory lung injury in COVID-19 and SARS[24,25]. Two recent studies found that S- and RBD-specific immunoglobulin G (IgG) antibodies in patients with COVID-19 have lower levels of fucosylation within their Fc domains[26,27]—a phenotype linked to higher affinity for FcγRIIIa, an activating Fc receptor (FcR) that mediates antibody-dependent cellular cytotoxicity. While this higher affinity can be beneficial in some cases via more vigorous FcγRIIIa-mediated effector functions[28,29], non-neutralizing IgG antibodies against dengue virus that were afucosylated were associated with more severe disease outcomes[30]. Larsen et al. further show that S-specific IgG in patients with both COVID-19 and acute respiratory distress syndrome had lower levels of fucosylation compared to patients who had asymptomatic or mild infections[26]. Whether the lower levels of fucosylation of SARS-CoV-2-specific antibodies directly contributed to COVID-19 immunopathology remains to be determined.

Importantly, SARS-CoV-2 has not been shown to productively infect macrophages[31,32]. Thus, available data suggest that the most probable ADE mechanism relevant to COVID-19 pathology is the formation of antibody–antigen immune complexes that leads to excessive activation of the immune cascade in lung tissue (Fig. 1).

Evidence of ADE in coronavirus infections in vitro

While ADE has been well documented in vitro for a number of viruses, including human immunodeficiency virus (HIV)[33,34], Ebola[35,36], influenza[37] and flaviviruses[38], the relevance of in vitro ADE for human coronaviruses remains less clear. Several studies have shown increased uptake of SARS-CoV and MERS-CoV virions into FcR-expressing monocytes or macrophages in vitro[32,39–42]. Yip et al. found enhanced uptake of SARS-CoV and S-expressing pseudoviruses into monocyte-derived macrophages mediated by FcγRIIa and anti-S serum antibodies[32]. Similarly, Wan et al. showed that a neutralizing monoclonal antibody (mAb) against the RBD of MERS-CoV increased the uptake of virions into macrophages and various cell lines transfected with FcγRIIa[39]. However, the fact that antigen-specific antibodies drive phagocytic uptake is unsurprising, as monocytes and macrophages can mediate antibody-dependent phagocytosis via FcγRIIa for viral clearance, including for influenza[43]. Importantly, macrophages in infected mice contributed to antibody-mediated clearance of SARS-CoV[44]. While MERS-CoV has been found to productively infect macrophages[45], SARS-CoV infection of macrophages is abortive and does not alter the pro-inflammatory cytokine gene expression profile after antibody-dependent uptake[41,42]. Findings to date argue against macrophages as productive hosts of SARS-CoV-2 infection[31,32].

ADE in human coronavirus infections

No definitive role for ADE in human coronavirus diseases has been established. Concerns were first raised for ADE in patients with SARS when seroconversion and neutralizing antibody responses were found to correlate with clinical severity and mortality[46]. A similar finding in patients with COVID-19 was reported, with higher antibody titres against SARS-CoV-2 being associated with

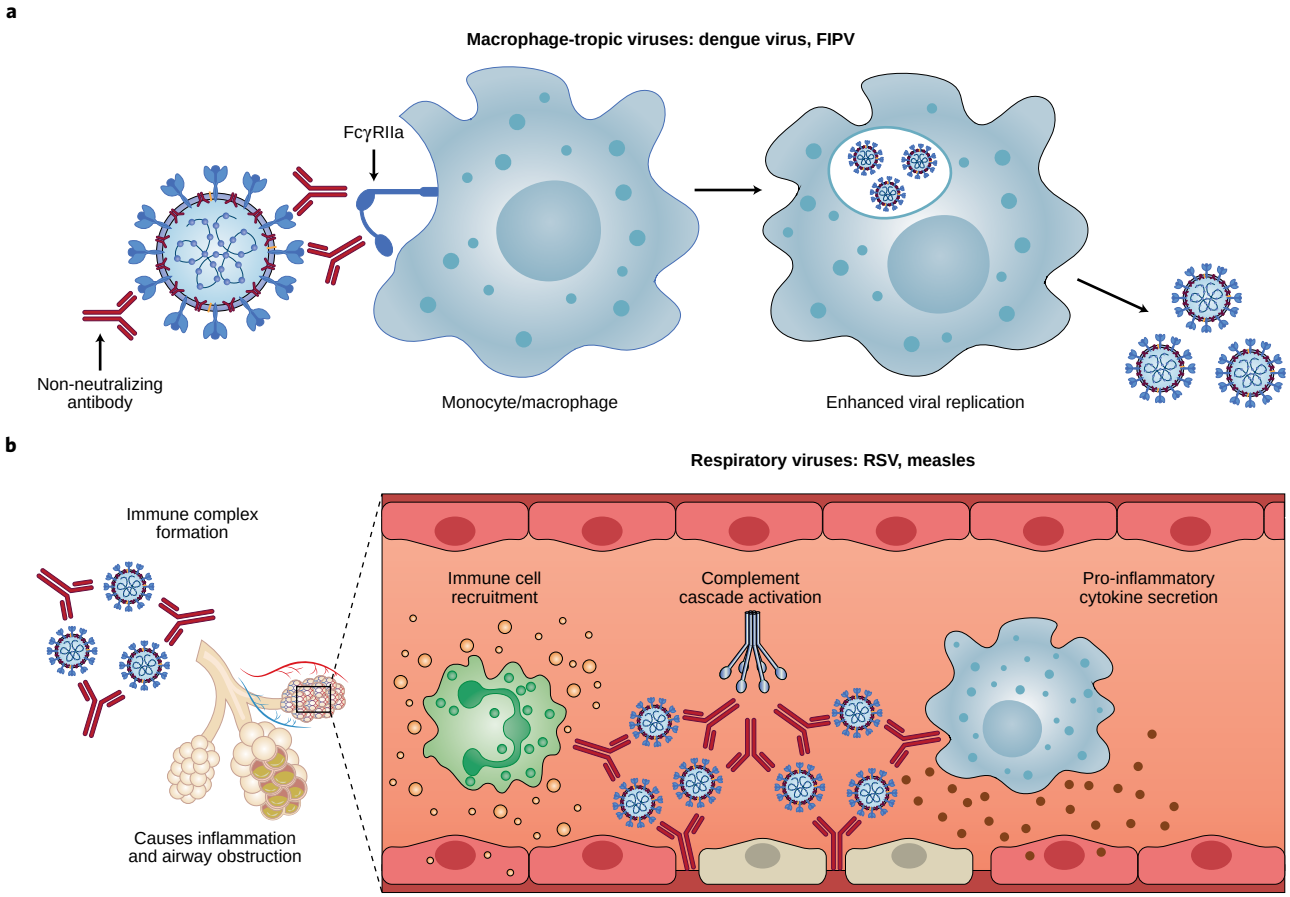

Fig. 1 | Two main ADE mechanisms in viral disease. a, For macrophage-tropic viruses such as dengue virus and FIPV, non-neutralizing or sub-neutralizing antibodies cause increased viral infection of monocytes or macrophages via FcγRIIa-mediated endocytosis, resulting in more severe disease. **b**, For non-macrophage-tropic respiratory viruses such as RSV and measles, non-neutralizing antibodies can form immune complexes with viral antigens inside airway tissues, resulting in the secretion of pro-inflammatory cytokines, immune cell recruitment and activation of the complement cascade within lung tissue. The ensuing inflammation can lead to airway obstruction and can cause acute respiratory distress syndrome in severe cases. COVID-19 immunopathology studies are still ongoing and the latest available data suggest that human macrophage infection by SARS-CoV-2 is unproductive. Existing evidence suggests that immune complex formation, complement deposition and local immune activation present the most likely ADE mechanisms in COVID-19 immunopathology. Figure created using BioRender.com.

more severe disease[47]. One simple hypothesis is that greater antibody titres in severe COVID-19 cases result from higher and more prolonged antigen exposure due to higher viral loads[48,49]. However, a recent study showed that viral shedding in the upper respiratory tract was indistinguishable between patients with asymptomatic and symptomatic COVID-19 (ref. [50]). Symptomatic patients showed higher anti-SARS-CoV-2 antibody titres and cleared the virus from the upper respiratory tract more quickly, contradicting a simpler hypothesis that antibody titres are simply caused by higher viral loads. Other studies showed that anti-SARS-CoV-2 T-cell responses could be found at high levels in mild and asymptomatic infections[51,52]. Taken together, the data suggest that strong T-cell responses can be found in patients with a broad range of clinical presentations, whereas strong antibody titres are more closely linked to severe COVID-19. One important caveat is that viral shedding was measured in the upper respiratory tract rather than in the lower respiratory tract[50]. The lower respiratory tract is likely more important for severe COVID-19 lung pathology, and it is unclear how closely SARS-CoV-2 viral shedding in the upper and lower respiratory tracts correlate throughout the disease course.

Beyond the host response to new SARS-CoV-2 infections, the potential of pre-existing antibodies against other human coronavirus strains to mediate ADE in patients with COVID-19 is another possible concern[53]. Antibodies elicited by coronavirus strains endemic in human populations (such as HKU1, OC43, NL63 and 229E) could theoretically mediate ADE by facilitating cross-reactive recognition of SARS-CoV-2 in the absence of viral neutralization. Preliminary data show that antibodies from SARS-CoV-2-naïve donors who had high reactivity to seasonal human coronavirus strains were found to have low levels of cross-reactivity against the nucleocapsid and S2 subunit of SARS-CoV-2 (ref. [54]). Whether such cross-reactive antibodies can contribute to clinical ADE of SARS-COV-2 remains to be addressed.

Risk of ERD for SARS-CoV-2 vaccines

Safety concerns for SARS-CoV-2 vaccines were initially fuelled by mouse studies that showed enhanced immunopathology, or ERD, in animals vaccinated with SARS-CoV following viral challenge[55–58]. The observed immunopathology was associated with Th2-cell-biased responses[55] and was largely against the nucleocapsid protein[56,58]. Importantly, immunopathology was not observed in challenged mice following the passive transfer of nucleocapsid-specific immune serum[56], confirming that the enhanced disease could not be replicated using the serum volumes transferred. Similar studies

using inactivated whole-virus or viral-vector-based vaccines for SARS-CoV or MERS-CoV resulted in immunopathology following viral challenge[59–61], which were linked to Th2-cytokine-biased responses[55] and/or excessive lung eosinophilic infiltration[57]. Rational adjuvant selection ensures that Th1-cell-biased responses can markedly reduce these vaccine-associated ERD risks. Candidate SARS-CoV vaccines formulated with either alum, CpG or Advax (a delta inulin-based adjuvant) found that while the Th2-biased responses associated with alum drove lung eosinophilic immunopathology in mice, protection without immunopathology and a more balanced Th1/Th2 response were induced by Advax[62]. Hashem et al. showed that mice vaccinated with an adenovirus 5 viral vector expressing MERS-CoV S1 exhibited pulmonary pathology following viral challenge, despite conferring protection. Importantly, the inclusion of CD40L as a molecular adjuvant boosted Th1 responses and prevented the vaccine-related immunopathology[63].

Should it occur, ERD caused by human vaccines will first be observed in larger phase II and/or phase III efficacy trials that have sufficient infection events for statistical comparisons between the immunized and placebo control study arms. Safety profiles of COVID-19 vaccines should be closely monitored in real time during human efficacy trials, especially for vaccine modalities that may have a higher theoretical potential to cause immunopathology (such as inactivated whole-virus formulations or viral vectors)[64,65].

Risk of ADE for SARS-CoV-2 vaccines

Evidence for vaccine-induced ADE in animal models of SARS-CoV is conflicting, and raises potential safety concerns. Liu et al. found that while macaques immunized with a modified vaccinia Ankara viral vector expressing the SARS-CoV S protein had reduced viral replication after challenge, anti-S IgG also enhanced pulmonary infiltration of inflammatory macrophages and resulted in more severe lung injury compared to unvaccinated animals[66]. They further showed that the presence of anti-S IgG prior to viral clearance skewed the wound-healing response of macrophages into a pro-inflammatory response. In another study, Wang et al. immunized macaques with four B-cell peptide epitopes of the SARS-CoV S protein and demonstrated that while three peptides elicited antibodies that protected macaques from viral challenge, one of the peptide vaccines induced antibodies that enhanced infection in vitro and resulted in more severe lung pathology in vivo[67].

In contrast, to determine whether low titres of neutralizing antibodies could enhance infection in vivo, Luo et al. challenged rhesus macaques with SARS-CoV nine weeks post-immunization with an inactivated vaccine, when neutralizing antibody titres had waned below protective levels[68]. While most immunized macaques became infected following viral challenge, they had lower viral titres compared to placebo controls and did not show higher levels of lung pathology. Similarly, Qin et al. showed that an inactivated SARS-CoV vaccine protected cynomolgus macaques from viral challenge and did not result in enhanced lung immunopathology, even in macaques with low neutralizing antibody titres[69]. A study in hamsters demonstrated that despite enhanced in vitro viral entry into B cells via FcγRII, animals vaccinated with the recombinant SARS-CoV S protein were protected and did not show enhanced lung pathology following viral challenge[70].

SARS-CoV immunization studies in animal models have thus produced results that vary greatly in terms of protective efficacy, immunopathology and potential ADE, depending on the vaccine strategy employed. Despite this, vaccines that elicit neutralizing antibodies against the S protein reliably protect animals from SARS-CoV challenge without evidence of enhancement of infection or disease[71–73]. These data suggest that human immunization strategies for SARS-CoV-2 that elicit high neutralizing antibody titres have a high chance of success with minimal risk of ADE. For example, subunit vaccines that can elicit S-specific neutralizing antibodies should present lower ADE risks (especially against S stabilized in the prefusion conformation, to reduce the presentation of non-neutralizing epitopes[8]). These modern immunogen design approaches should reduce potential immunopathology associated with non-neutralizing antibodies.

Vaccines with a high theoretical risk of inducing pathologic ADE or ERD include inactivated viral vaccines, which may contain non-neutralizing antigen targets and/or the S protein in non-neutralizing conformations, providing a multitude of non-protective targets for antibodies that could drive additional inflammation via the well-described mechanisms observed for other respiratory pathogens. However, it is encouraging that a recent assessment of an inactivated SARS-CoV-2 vaccine elicited strong neutralizing antibodies in mice, rats and rhesus macaques, and provided dose-dependent protection without evidence of enhanced pathology in rhesus macaques[74]. Going forward, increased vaccine studies in the Syrian hamster model may provide critical preclinical data, as the Syrian hamster appears to replicate human COVID-19 immunopathology more closely than rhesus macaque models[75].

ADE and recombinant antibody interventions

The discovery of mAbs against the SARS-CoV-2 S protein is progressing rapidly. Recent advances in B-cell screening and antibody discovery have enabled the rapid isolation of potent SARS-CoV-2 neutralizing antibodies from convalescent human donors[76,77] and immunized animal models[78], and through re-engineering previously identified SARS-CoV antibodies[79]. Many more potently neutralizing antibodies will be identified in the coming weeks and months, and several human clinical trials are ongoing in July 2020. Human trials will comprise both prophylactic and therapeutic uses, both for single mAbs and cocktails. Some human clinical trials are also incorporating FcR knockout mutations to further reduce ADE risks[80]. Preclinical data suggest a low risk of ADE for potently neutralizing mAbs at doses substantially above the threshold for neutralization, which protected mice and Syrian hamsters against SARS-CoV-2 challenge without enhancement of infection or disease[81,82]. ADE risks could increase in the time period where mAb concentrations have waned below a threshold for protection (which is analogous to the historical mother–infant data that provided important clinical evidence for ADE in dengue[83]). The sub-protective concentration range will likely occur several weeks or months following mAb administration, when much of the initial drug dose has cleared the body. Notably, Syrian hamsters given low doses of an RBD-specific neutralizing mAb prior to challenge with SARS-CoV-2 showed a trend for greater weight loss than control animals[82], though differences were not statistically significant and the low-dose animals had lower viral loads in the lung compared to control animals. Non-neutralizing mAbs against SARS-CoV-2 could also be administered before or after infection in a hamster model to determine whether non-neutralizing antibodies enhance disease. Passive transfer of mAbs at various time points after infection (for example, in the presence of high viral loads during peak infection) could also address the question of whether immune complex formation and deposition results in the enhancement of disease and lung immunopathology. If ADE of neutralizing or non-neutralizing mAbs is a concern, the Fc portion of these antibodies could be engineered with mutations that abrogate FcR binding[80]. Animal studies can help to inform whether Fc-mediated effector functions are crucial in preventing, treating or worsening SARS-CoV-2 infection, in a similar way to previous studies of influenza A and B infection in mice[84,85] and simian-HIV infection in macaques[86,87]. An important caveat for testing human mAbs in animal models is that human antibody Fc regions may not interact with animal FcRs in the same way as human FcRs[88]. Whenever possible, antibodies used for preclinical ADE studies will require species-matched Fc regions to appropriately model Fc effector function.

ADE and convalescent plasma interventions

Convalescent plasma (CP) therapy has been used to treat patients with severe disease during many viral outbreaks in the absence of effective antiviral therapeutics. It can offer a rapid solution for therapies until molecularly defined drug products can be discovered, evaluated and produced at scale. While there is a theoretical risk that CP antibodies could enhance disease via ADE, case reports in SARS-CoV and MERS-CoV outbreaks showed that CP therapy was safe and was associated with improved clinical outcomes[89,90]. One of the largest studies during the SARS outbreak reported the treatment of 80 patients with SARS in Hong Kong[91]. While there was no placebo control group, no CP-associated adverse effects were detected and there was a higher discharge rate among patients treated earlier in infection. Several small studies of individuals with severe COVID-19 disease and a study of 5,000 patients with COVID-19 have shown that CP therapy appears safe and may improve disease outcomes[92–96], although the benefits appear to be mild[97]. However, it is difficult to determine whether CP therapy contributed to recovery as most studies to date were uncontrolled and many patients were also treated with other drugs, including antivirals and corticosteroids. The potential benefits of CP therapy in patients with severe COVID-19 is also unclear, as patients with severe disease may have already developed high antibody titres against SARS-CoV-2 (refs. [47,98]). CP has been suggested for prophylactic use in high-risk populations, including people with underlying risk factors, frontline healthcare workers and people with exposure to confirmed COVID-19 cases[99]. CP for prophylactic use may pose an even lower ADE risk compared to its therapeutic use, as there is a lower antigenic load associated with early viral transmission compared to established respiratory infection. As we highlighted above with recombinant mAbs, and as shown in historical dengue virus mother–infant data, the theoretical risk of ADE in CP prophylaxis is highest in the weeks following transfusion, when antibody serum neutralization titres fall to sub-protective levels. ADE risks in CP studies will be more difficult to quantify than in recombinant mAb studies because the precise CP composition varies widely across treated patients and treatment protocols, especially in CP studies that are performed as one-to-one patient–recipient protocols without plasma pooling.

To mitigate potential ADE risks in CP therapy and prophylaxis, plasma donors could be pre-screened for high neutralization titres. Anti-S or anti-RBD antibodies could also be purified from donated CP to enrich for neutralizing antibodies and to avoid the risks of ADE caused by non-neutralizing antibodies against other SARS-CoV-2 antigens. Passive infusion studies in animal models are helping to clarify CP risks in a well-controlled environment, both for prophylactic and therapeutic use. Key animal studies (especially in Syrian hamsters, and ideally with hamster-derived CP for matched antibody Fc regions) and human clinical safety and efficacy results for CP are now emerging contemporaneously. These preclinical and clinical data will be helpful to deconvolute the risk profiles for ADE versus other known severe adverse events that can occur with human CP, including transfusion-related acute lung injury[96,100].

Conclusion

ADE has been observed in SARS, MERS and other human respiratory virus infections including RSV and measles, which suggests a real risk of ADE for SARS-CoV-2 vaccines and antibody-based interventions. However, clinical data has not yet fully established a role for ADE in human COVID-19 pathology. Steps to reduce the risks of ADE from immunotherapies include the induction or delivery of high doses of potent neutralizing antibodies, rather than lower concentrations of non-neutralizing antibodies that would be more likely to cause ADE.

Going forwards, it will be crucial to evaluate animal and clinical datasets for signs of ADE, and to balance ADE-related safety risks against intervention efficacy if clinical ADE is observed. Ongoing animal and human clinical studies will provide important insights into the mechanisms of ADE in COVID-19. Such evidence is sorely needed to ensure product safety in the large-scale medical interventions that are likely required to reduce the global burden of COVID-19.

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Author contributions

W.S.L., A.K.W., S.J.K. and B.J.D. drafted the manuscript, edited the draft and prepared the final manuscript, which was approved by all co-authors.

Competing interests

The authors declare no competing interests.

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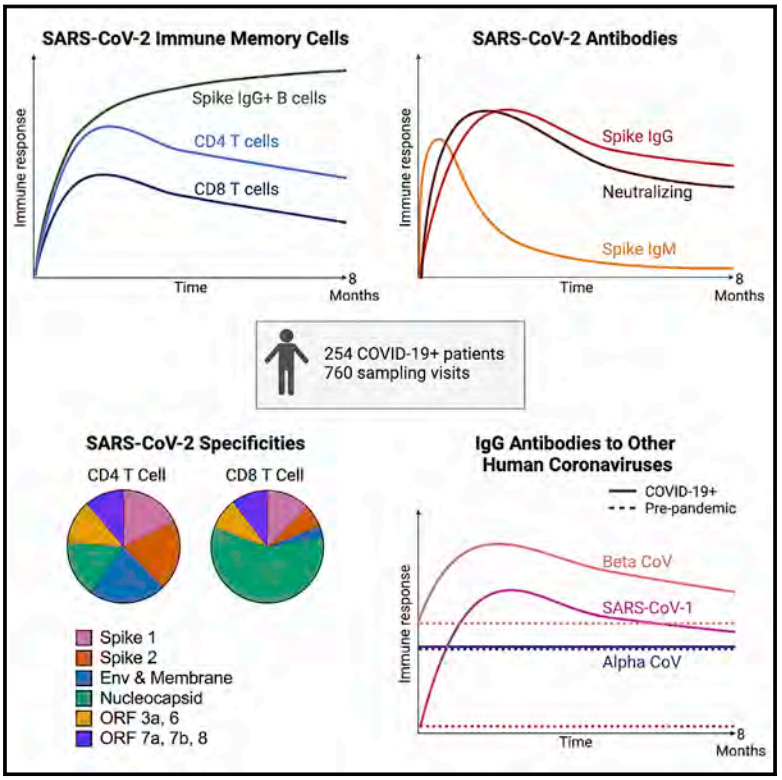
Article

Longitudinal analysis shows durable and broad immune memory after SARS-CoV-2 infection with persisting antibody responses and memory B and T cells

Graphical abstract



Highlights

- Most recovered COVID-19 patients mount broad, durable immunity after infection
- Neutralizing antibodies show a bi-phasic decay with half-lives >200 days
- Spike IgG+ memory B cells increase and persist post-infection
- Durable polyfunctional CD4 and CD8 T cells recognize distinct viral epitope regions

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In brief

Cohen et al. evaluate immune responses longitudinally in 254 COVID-19 patients over 8 months. SARS-CoV-2-specific binding and neutralizing antibodies exhibit biphasic decay, suggesting long-lived plasma cell generation. Memory B cells remain stable; CD4 and CD8 memory T cells are polyfunctional. Thus, broad and effective immunity may persist long-term following COVID-19.

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Article

Longitudinal analysis shows durable and broad immune memory after SARS-CoV-2 infection with persisting antibody responses and memory B and T cells

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SUMMARY

Ending the COVID-19 pandemic will require long-lived immunity to SARS-CoV-2. Here, we evaluate 254 COVID-19 patients longitudinally up to 8 months and find durable broad-based immune responses. SARS-CoV-2 spike binding and neutralizing antibodies exhibit a bi-phasic decay with an extended half-life of >200 days suggesting the generation of longer-lived plasma cells. SARS-CoV-2 infection also boosts antibody titers to SARS-CoV-1 and common betacoronaviruses. In addition, spike-specific IgG+ memory B cells persist, which bodes well for a rapid antibody response upon virus re-exposure or vaccination. Virus-specific CD4+ and CD8+ T cells are polyfunctional and maintained with an estimated half-life of 200 days. Interestingly, CD4+ T cell responses equally target several SARS-CoV-2 proteins, whereas the CD8+ T cell responses preferentially target the nucleoprotein, highlighting the potential importance of including the nucleoprotein in future vaccines. Taken together, these results suggest that broad and effective immunity may persist long-term in recovered COVID-19 patients.

INTRODUCTION

The COVID-19 pandemic caused by the rapid spread of SARS-CoV-2, a novel betacoronavirus, continues to cause significant morbidity and mortality. The induction of effective early immune control of SARS-CoV-2 and durable immune memory is critical to prevent severe disease and to protect upon re-exposure. SARS-CoV-2 infection induces polyclonal humoral and cellular responses targeting multiple viral proteins described in cross-sectional and longitudinal studies.[1] More comprehensive, quantitative analyses with extensive serial sampling in larger numbers of COVID-19 patients are limited and could resolve some conflicting views about the durability of humoral immunity. Importantly, defining the frequency, immune function, and specificity of the antibodies; memory B and T cell responses among COVID-19 patients; and identifying when they appear and how long they persist can provide understanding of the integral components for long-lived immunity to SARS-CoV-2 and potentially other human coronaviruses that emerge in the future.[2]

We initiated two prospective COVID-19 patient cohorts in Seattle and Atlanta during the first surge of the pandemic to investigate long-term immunity to SARS-CoV-2. Among 254 COVID-19 patients enrolled and frequently sampled, we identify binding and neutralizing antibodies to SARS-CoV-2 as well as antigen-specific B and T cells elicited early after infection, define their specificities, quantify the extent of antibody boosting of cross-reactive

responses to other coronaviruses, and further characterize the decay rate and durability of these immune parameters over 250 days. We employ highly standardized or validated assays that are also being used to evaluate immunity in recent and ongoing clinical vaccine trials.[3-5] This in-depth longitudinal study demonstrates that durable immune memory persists in most COVID-19 patients, including those with mild disease, and serves as a framework to define and predict long-lived immunity to SARS-CoV-2 after natural infection. This investigation will also serve as a benchmark for immune memory induced in humans by SARS-CoV-2 vaccines.

RESULTS

COVID-19 study population

COVID-19-confirmed patients were recruited into our longitudinal study of SARS-CoV-2 specific B and T cell memory after infection. A total of 254 patients were enrolled at two sites, Atlanta and Seattle, starting in April 2020 and returned for follow up visits over a period of 250 days. We were able to collect blood samples at 2–3 time points from 165 patients and at 4–7 time points from another 80 patients, which allowed us to perform a longitudinal analysis of SARS-CoV-2-specific B and T cell responses on a large number of infected patients. The demographics and baseline characteristics of this cohort are described in Table S1. The study group was 55% female and 45% male and between 18 and 82 years old (median, 48.5 years). Based on World Health Organization (WHO) guidelines of disease severity, 71% of study participants exhibited mild disease, 24% had moderate disease, and 5% experienced severe disease.

Antibody responses to SARS-CoV-2 spike protein show a bi-phasic decay with an extended half-life

Binding antibodies to the SARS-CoV-2 full-length spike protein, to the receptor binding domain (RBD), and to the N-terminal domain (NTD) of the spike protein were assessed in COVID-19 patients (n = 222) over a period of 8 months post symptom onset. We included healthy individuals age 18–42 years as negative controls whose longitudinal blood samples were collected before the emergence of the COVID-19 pandemic. These pre-pandemic samples (n = 51) were from recipients of either the seasonal inactivated influenza vaccine (n = 27, collected from 2014-2018) or the live yellow fever virus (YFV-17D) vaccine (n = 24, collected from 2005–2007). The Mesoscale multiplex assay was used to measure IgG, IgA, and IgM antibody responses to SARS-CoV-2 proteins in the COVID-19 patients and in the pre-pandemic healthy controls.

The magnitude of serum IgG antibodies binding to the SARS-CoV-2 spike protein increased in 92% of COVID-19 convalescent participants (n = 222) relative to pre-pandemic controls (Figure 1A). The IgG responses to SARS-CoV-2 spike, RBD, and NTD declined over time with half-lives of 126 (95% confidence interval [95% CI] [107, 154]), 116 (95% CI [97, 144]), and 130 (95% CI [110, 158]) days, respectively, as estimated by an exponential decay model (Figures 1A–1C and S1A). We also estimated antibody waning using a power law model, which models a scenario in which the rate of antibody decay slows over time. The power law model produced a better fit for the decay of the SARS-CoV-2 spike, RBD, and NTD binding IgG antibodies (DAICs > 10), suggesting that spike-specific antibodies plateau over time. Because the decay rate changes over time, the half-life is predicted to change over time as well; therefore, we used the power law model to estimate the half-lives at 120 days after symptom onset. The power law estimated half-lives for the IgG antibody responses to spike ($t_{1/2}$ = 238 days), RBD ($t_{1/2}$ = 209 days), and NTD ($t_{1/2}$ = 244 days) were longer than those estimated by the exponential decay model (Figures S1A and S1C), indicating that the concentration of these IgG antibodies may be starting to stabilize. IgA (Figures 1D–1F) and IgM (Figures 1G–1I) antibodies reactive to the SARS-CoV-2 spike also increased after SARS-CoV-2 infection but were detected at lower levels and declined faster than the SARS-CoV-2-reactive IgG antibodies. As expected, spike-binding IgM decayed more rapidly than spike-binding IgA and IgG. Taken together, these results show that antibody responses, especially IgG antibody, were not only durable in the vast majority of patients in the 250 day period, but also that the bi-phasic decay curve suggests the generation of longer lived plasma cells producing antibody to the SARS-CoV-2 spike protein.

We also examined the antibody response to the SARS-CoV-2 nucleocapsid protein in these infected patients. As expected, the COVID-19 patients showed higher levels of antibody to the nucleocapsid protein compared to the pre-pandemic healthy controls (Figure S2). However, the nucleocapsid-specific antibodies declined with a much shorter half-life of 63 days (95% CI [58, 70]) compared to the spike protein antibodies (Figures S1A–S1C). Also, the nucleocapsid reactive IgG decay rate was best fit by the exponential model and not the power law model in contrast to what we observed with the spike IgG antibody decay rate (Figure S1A). Thus, the nucleocapsid reactive IgG not only declined much faster but also showed less evidence of stabilizing antibody levels, consistent with a response driven disproportionately by short-lived antibody secreting cells – at least at this stage of the immune response.

Stable and long-lived antibody responses to common human alpha- and betacoronaviruses in pre-pandemic healthy controls

We were interested in determining if SARS-CoV-2 infection had any effect on the levels of antibody to the circulating human alpha- and betacoronaviruses. As a prelude to this question, we first examined antibody levels to the spike protein of the two circulating alphacoronaviruses (229E and NL63) and the two betacoronaviruses (HKU1 and OC43) in our pre-pandemic samples. As shown in Figure 2, all 51 pre-pandemic samples had clearly detectable levels of IgG and IgA antibodies to the spike proteins of the four human coronaviruses. This is the expected result since seropositivity to these coronaviruses is very high in the adult population, but what was quite interesting was the remarkable stability of these antibody responses over a 200-day period in the pre-pandemic serum samples (shown as red lines in Figure 2). These were essentially flat lines with no decline in the antibody levels and question the prevailing belief that antibody responses to the endemic coronaviruses are short-lived.[6-8] While some occasional boosting of these childhood-acquired coronavirus infections cannot be ruled out, these data showing such stable antibody titers are best explained by

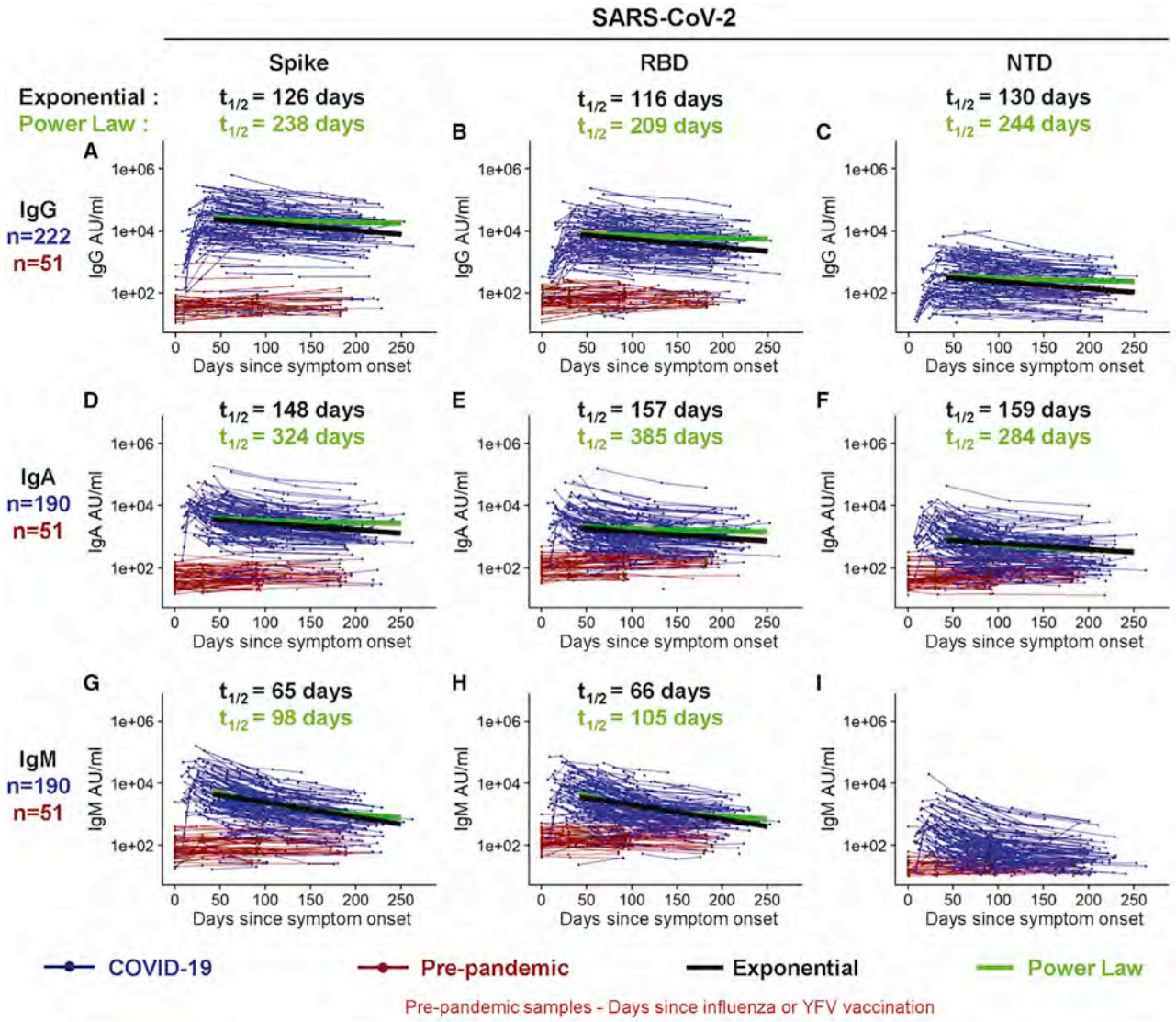

Figure 1. Longitudinal SARS-CoV-2 spike-binding antibody responses

IgG (A–C), IgA (D–F), and IgM (G–I) antibodies reactive to SARS-CoV-2 spike (A, D, G); spike receptor binding domain (RBD, [B, E, and H]), and the spike N-terminal domain (NTD, [C, F, and I]) were measured in triplicate by an electrochemiluminescent multiplex immunoassay and reported as arbitrary units per ml (AU/mL) as normalized by a standard curve. Longitudinal antibody titers of COVID-19 patients (in blue, n = 222 COVID-19+ for IgG; n = 190 COVID-19+ for IgA and for IgM) are plotted over days since symptom onset, whereas longitudinal pre-pandemic donor samples (in red, n = 51 for IgG, IgA, and IgM) were collected in the course of a non-SARS-CoV-2 vaccine study before 2019 and plotted over days since immunization. IgG decay curves and half-lives estimated by an exponential decay model are shown in black, and the decay curves and half-lives at day 120 post symptom onset estimated by a power law model are shown in green.

the persistence of long-lived plasma cells in the bone marrow many years after infection.[9-13]

COVID-19 infection results in increased levels of antibodies to two common human betacoronaviruses (HKU1 and OC43) and to SARS-CoV-1

We next examined if SARS-CoV-2 infection had any impact on the levels of antibodies to the other human coronaviruses. We measured IgG, IgA, and IgM antibody binding to the spike proteins of other known human coronaviruses in the COVID-19 patients (n = 222 for IgG and n = 190 for IgA and IgM) and compared these data to the 51 pre-pandemic healthy donor samples. In the COVID-19 patients, IgG and IgA antibodies to the alphacoronaviruses 229E and NL63 did not show any significant changes compared to the antibody levels in the pre-pandemic healthy controls (Figures 2A, 2B, 2F, and 2G; Figures S1C and S1D). In contrast, the IgG and IgA antibodies to betacoronaviruses HKU1 and OC43 were substantially elevated in COVID-19 patients relative to pre-pandemic controls (Figures 2C, 2D, 2H, and 2I; Figures S1C and S1D; $p < 0.0001$). After this boost, HKU1 and OC43 IgG antibody levels declined with estimated half-lives of 288 (95% CI [235, 372]) and 212 (95% CI [176, 268]) days, respectively (exponential decay

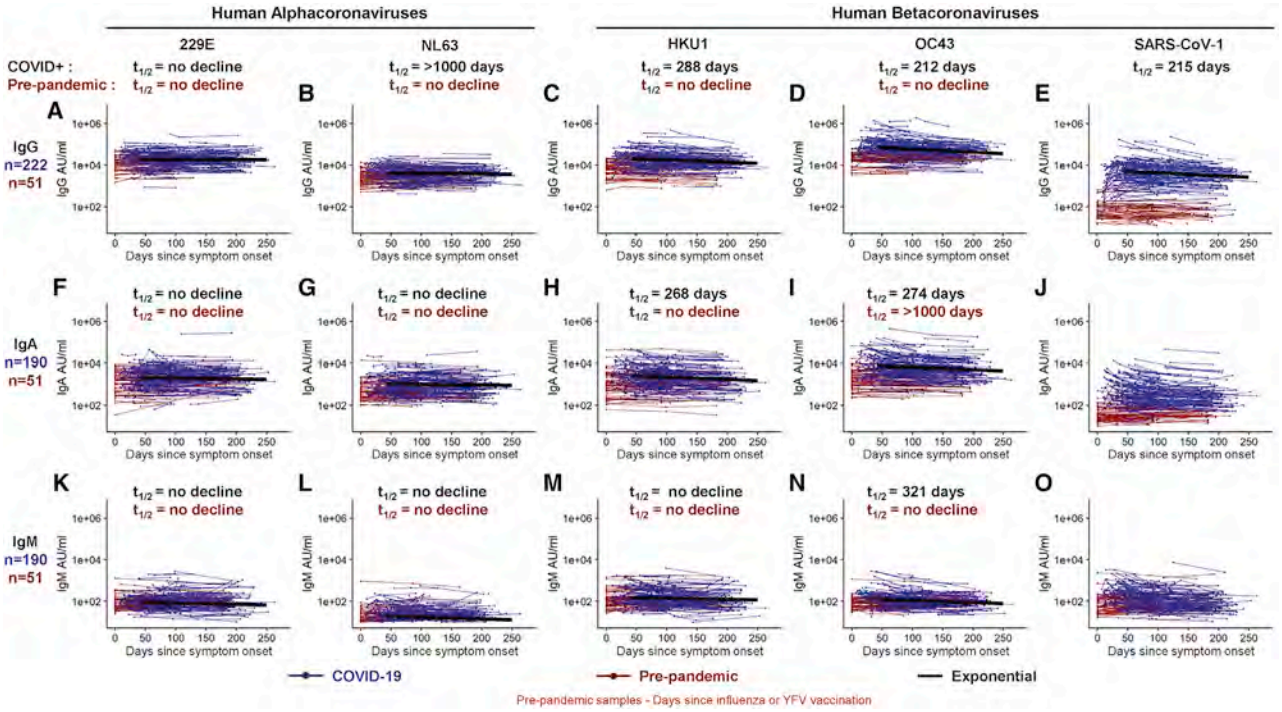

Figure 2. Longitudinal binding antibody responses to other coronavirus spike proteins

IgG (A–E), IgA (F–J), and IgM (K–O) antibody responses in sera collected from COVID-19+ patients (in blue, n = 222 for IgG; n = 190 for IgA and IgM) and pre-pandemic donors (in red, n = 51 for IgG, IgA and IgM) that were measured to 229E spike (A, F, and K), NL63 spike (B, G, and L), HKU1 spike (C, H, and M), OC43 spike (D, I, and N), and the SARS-CoV-1 spike protein (E, J, and O) in triplicate. Longitudinal antibody titers of COVID-19 patients are plotted over days since symptom onset, whereas longitudinal pre-pandemic donor samples were collected in the course of a non-SARS-CoV-2 vaccine study before 2019 and plotted over days since immunization. Antibody responses were measured by an electrochemiluminescent multiplex immunoassay and reported as arbitrary units per ml (AU/mL) as normalized by a standard curve. IgG decay curves and half-lives estimated by an exponential decay model are shown in black. There was no significant decline in IgG reactive to endemic alpha and betacoronaviruses in longitudinal samples collected in healthy donors before the pandemic (red, [A–D]).

model). IgM levels to common betacoronaviruses HKU1 and OC43 were low in both pre-pandemic controls and COVID-19 patients (Figures 2M and 2N). While pre-existing exposure and antibodies against HKU1 and OC43 betacoronaviruses are common in adults, pre-existing SARS-CoV-1 exposure is rare and antibody levels to SARS-CoV-1 spike protein were very low (essentially negative) in the pre-pandemic healthy controls. However, SARS-CoV-1 spike-reactive antibodies increased significantly after SARS-CoV-2 infection. These increases were quite striking for IgG ($p = 0.0038$) and also IgA ($p = 0.0084$) and most likely represent cross-reactive antibodies directed to SARS-CoV-2 spike epitopes that are conserved between SARS-CoV-2 and SARS CoV-1[14]. These newly induced cross-reactive IgG antibodies generated after COVID-19 infection declined with an estimated half-life of 215 days (95% CI [168, 298]) (exponential decay model) (Figure 2). Taken together, these results show that people infected with SARS-CoV-2 may have also have some heightened immunity against the common human betacoronaviruses and more importantly against SARS-CoV-1.

Durable neutralizing antibody responses to SARS-CoV-2 in infected patients

Neutralizing antibodies were measured with a live virus focus reduction neutralization test that uses a recombinant SARS-CoV-2 virus expressing the fluorescent reporter gene mNeonGreen (FRNT-mNG) (Figure 3A). During the first 250 days post-symptom onset, $FRNT_{50}$ titers varied considerably between individuals and ranged from < 20 to 3726 (Figure 3A). Of the 183 individuals for whom longitudinal neutralization titers were assayed, 140 (77%) had at least one time point with neutralization titers above the limit of detection (> 20). Seventy-five percent (43/57) of COVID-19 patients generated serum neutralizing antibodies between 30–50 days after symptom onset and similarly 72% (48/67) had measurable titers between 180–263 days after symptom onset. Using an exponential decay model, we evaluated the kinetics of neutralizing antibody titers after day 42 and estimated a half-life of 150 days (95% CI [124, 226]). However, similar to the spike-reactive IgG binding antibodies, we hypothesized that the neutralizing antibody rate of decay may actually slow over time during the recovery period. To address this, we fit a power law to the data. The power law model fit significantly better than the exponential decay model (DAIC = 9) and estimated the half-life of neutralizing antibody responses at 120 days post-symptom onset to be 254 days (95% CI [183, 400]).

Next, we assessed the relationship between the levels of spike and RBD binding antibodies and SARS-CoV-2 neutralization. Figures 3B and C show the SARS-CoV-2 spike and RBD binding antibody response kinetics of the 183 participants for whom

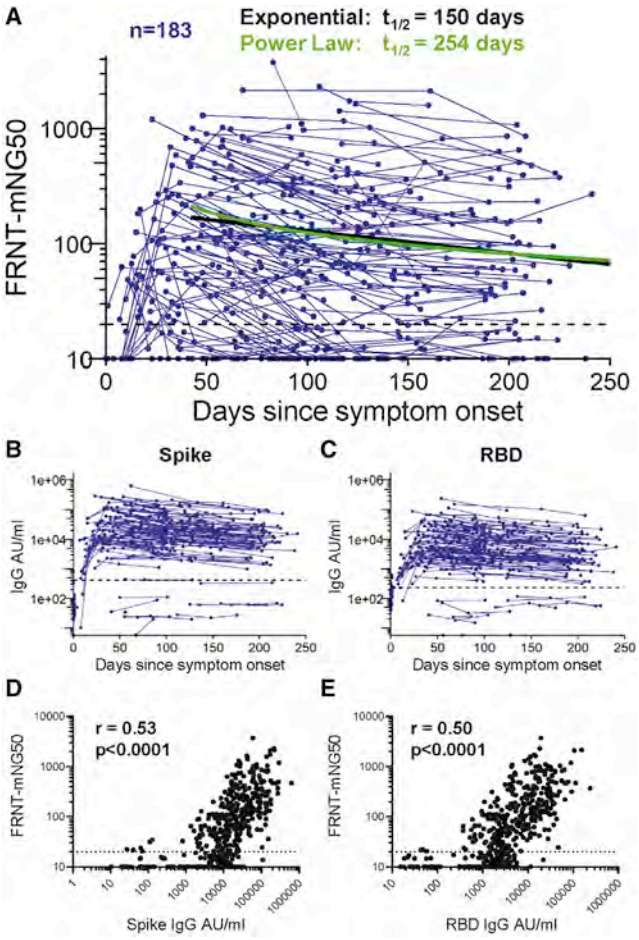

Figure 3. Neutralizing antibody responses to SARS-CoV-2

(A) *In vitro* serum neutralization antibody titers to SAR-CoV-2 were measured in duplicate by focus-reduction neutralization assay COVID-19 patients (n = 183). The limit of detection is indicated with a dashed line at FRNT-mNG$_{50}$ = 20. The half-life estimated by the exponential decay model (black) is 150 days, whereas the half-life estimated at day 120 using the power law model (green) is 254 days.

(B and C) IgG antibody titers reactive to SARS-CoV-2 spike (B) and RBD (C) of the matched 183 COVID-19 for whom neutralization titers were assessed. The geometric mean titer plus 3 standard deviations of pre-pandemic samples is indicated by a dashed line.

(D and E) SARS-CoV-2 spike (D) and RBD (E) reactive IgG levels correlated with neutralization titers at the matched time point (repeated-measures correlation, $p < 0.0001$). The limit of detection is indicated with a dashed line at FRNT-mNG$_{50}$ = 20.

neutralization titers were assessed. These exhibited a wide range of antibody binding levels ranging from non-responders (n = 11) who did not elicit antibody titers above those of pre-pandemic controls (defined as a COVID-19 patient titer below the mean pre-pandemic antibody titer plus three standard deviations, see dashed line on Figures 3B and 3C) to those with IgG levels > 200,000 AU/mL. Spike and RBD binding IgG levels correlated significantly with the neutralization titers (Figure 3D, E; $p < 0.0001$).

Taken together, our findings show that induction of neutralizing antibodies occurs in the majority of COVID-19 patients. These neutralizing antibodies can persist over the 8–9 month period following infection, and show a correlation with spike and RBD binding IgG.

SARS-CoV-2 spike and RBD-specific memory B cells increase for several months after infection and then plateau over 8 months

Memory B cells (MBC) are an important component of humoral immunity and contribute to viral control by generating antibody responses upon re-exposure to the pathogen. We used full-length spike and RBD antigen probes to quantify the frequencies of SARS-CoV-2 spike- and RBD-specific MBC in longitudinal PBMC samples from 111 COVID-19 patients (Figure 4) and from 29 pre-pandemic controls (Figures S3A and S3B). Our flow cytometric gating strategy to identify SARS-CoV-2-specific MBC and classify them as IgG, IgM, and IgA MBC isotypes is shown in Figure 4A.

Among the total MBC, the spike IgG+ MBCs were significantly increased in COVID-19 patients (n = 111; Figure 4B) in comparison to pre-pandemic controls (n = 29; Figure S3A) (median increase, 0.73% versus 0.02%; $p < 0.0001$). After a steep early expansion over the first 2-3 months, the spike IgG+ MBC persisted in COVID-19 patients with no decline out to 250 days post symptom onset. These findings (Figure 4B) are supported by a positive slope (0.004) from the model of the longitudinal spike IgG+ MBC responses after day 30 (95% CI [0.002, 0.006], $p < 0.001$; Figures S4A and S4B).

The spike IgM+ MBC appeared within the first 2 weeks post-symptom onset and quickly declined (Figures 4C and 4D). The decay continued after day 30 (slope = −0.007, 95% CI [-0.010, −0.005], $p < 0.001$). One month after symptom onset, 56% of spike MBC were IgG+, which increased to a peak of 80% at 5–6 months (Figure 4D). Circulating spike IgA+ MBC were also detectable in many subjects at low frequencies and without significant change over time (day 30–250: slope = 0.000, 95% CI [-0.002, 0.002], $p = 0.91$, Figure 4D).

Since the RBD contains the primary neutralizing epitopes on the spike, we also used an RBD-specific probe to characterize this subset of spike-specific memory B cells. Overall, approximately 20% of the spike IgG+ memory B cells targeted the RBD, which was consistent across subjects and time (Figures 4E and 4F). As expected, RBD+ IgM+ MBC emerged early in infection and subsequently switched to RBD+ IgG+ MBCs, which gradually increased during follow-up (day 30–250: slope = 0.004, 95% CI [0.002, 0.005], $p < 0.001$, Figure 4E). Thus, the maintenance of circulating spike- and RBD-specific IgG memory B cells suggests that these cells could be recruited for a rapid secondary response following re-exposure or vaccination.

Induction of durable and polyfunctional virus specific memory CD4+ and CD8+ T cells in infected patients

CD4+ T cells are critical for generation of high affinity antibody responses and can also have anti-viral effects. In addition, they provide help for CD8+ T cell responses, which are vital for killing infected cells and mediating viral clearance. Thus, we next examined virus-specific CD4+ and CD8+ T cell responses longitudinally in COVID-19 patients and uninfected controls using a high-dimensional, multi-parameter *ex vivo* intracellular cytokine staining (ICS)

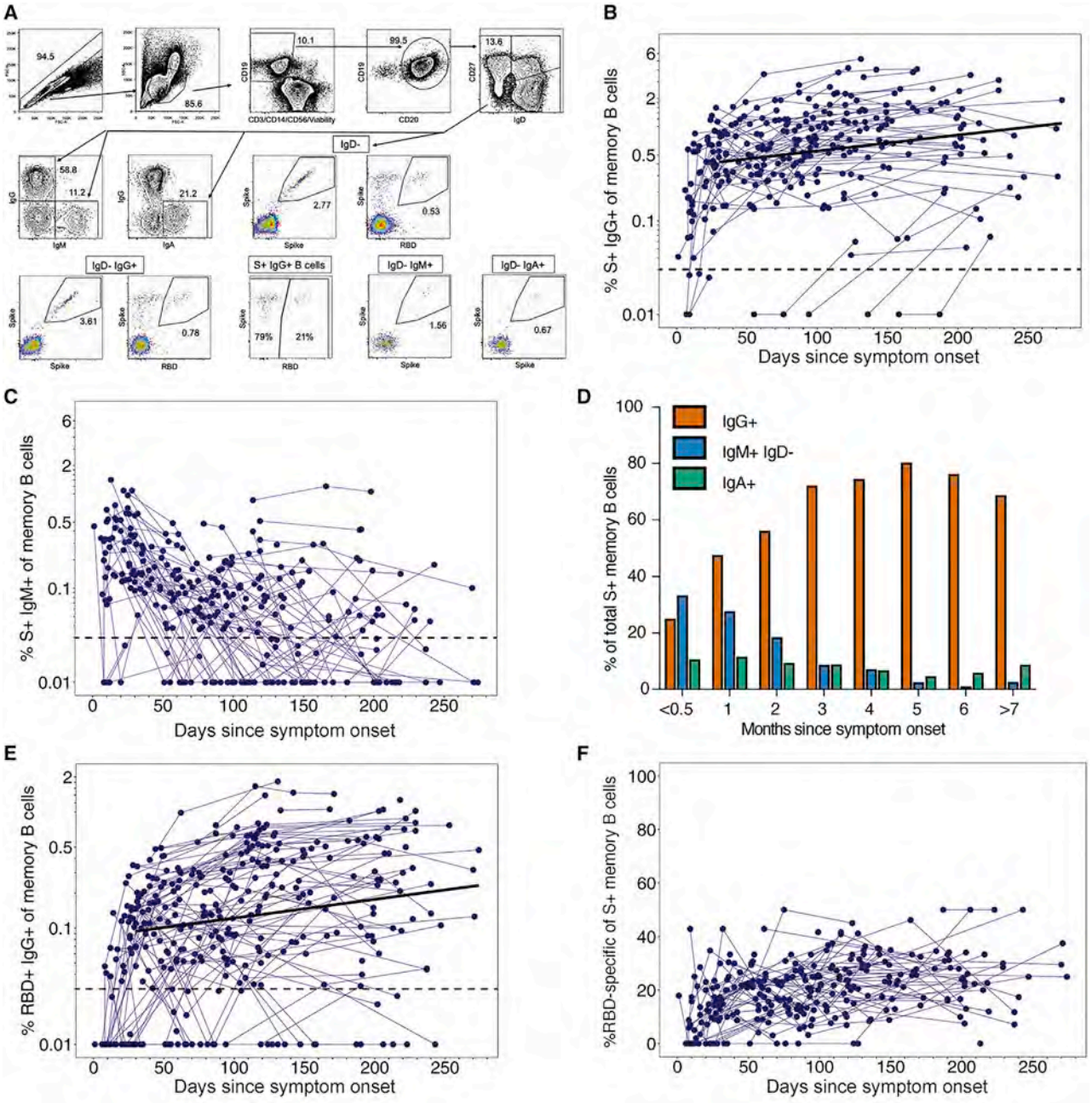

Figure 4. SARS-CoV-2 spike and RBD-specific memory B cells

(A) Representative memory B cell gating strategy is shown for identification of SARS-CoV-2 spike and RBD-specific IgD- IgG+, IgD- IgM+, and IgD- IgA+ memory B cells in PBMCs from a SARS-CoV-2 convalescent participant.

(B and C) The frequency of spike+ (B) IgG+ and (C) IgM+ memory B cells out of memory B cells (IgD- CD19+ CD20+) is displayed over time from initial symptom onset among SARS-CoV-2-infected subjects (n = 105 subjects; measured in singlet replicates). The dashed line indicates the limit of detection. The bold line represents the median fitted curve from a linear mixed effects model of post-day 30 responses.

(D) The median percent of spike+ memory B cells expressing IgG, IgM or IgA isotypes was assessed at monthly intervals post-symptom onset.

(E) The frequency of RBD+ IgG+ of memory B cells over time (n = 141).

(F) The proportion of S+ IgG+ memory B cells that are specific for the receptor binding domain are depicted over time.

assay. The assay is sensitive, precise, and specific for detection of antigen-specific T cells expressing multiple cytokines and effector molecules following a short-term (6 h) stimulation with peptide pools. Our lab developed and validated the assay, and we are currently using the method to quantitate Th1/Th2 CD4+ and CD8+ T cell responses in SARS-CoV-2 vaccine trials. Here,

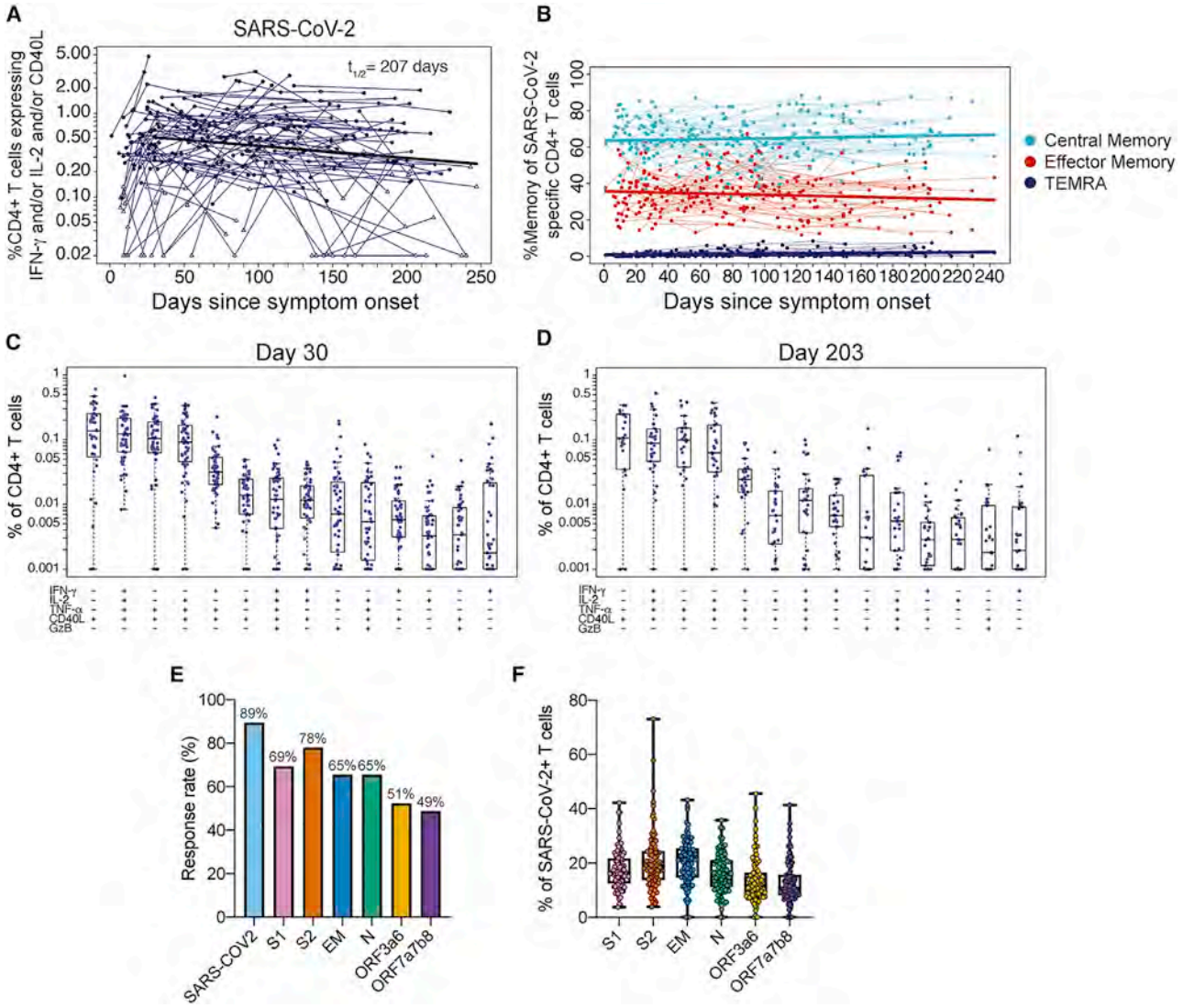

Figure 5. CD4+ T cell responses to SARS-CoV-2 antigens

(A) The sum of background-subtracted CD4+ T cells expressing *ex vivo* IFN-g, IL-2 and/or CD40L to peptide pools spanning SARS-CoV-2 structural proteins: S1, S2, envelope (E), membrane (M), nucleocapsid (N), and the following ORFs: 3a, 3b, 6, 7a, 7b, and 8 (n = 114; tested in singlets) for each individual/time point. Each sample that is "positive" (by MIMOSA) for at least one SARS-CoV-2 antigen is indicated by a solid circle, whereas samples that are "negative" for all of the SARS-CoV-2 antigens at that time point are indicated by open triangles. The bold line represents the median fitted curve from a nonlinear mixed effects model of post-day 30 responses among those with a positive response at $\geq$1 time point; $t_{1/2}$ is the median half-life estimated from the median slope, with 95% CI [104, 411].

(B) The proportion of SARS-CoV-2-specific CD4+ T cells expressing a specific memory phenotype over time: central memory (CCR7+ CD45RA-), effector memory (CCR7- CD45RA-), or T_{EMRA} (CCR7- CD45RA+); restricted to positive responders.

(C and D) Polyfunctionality of SARS-CoV-2-specific CD4+ T cells are shown at (C) 21-60 days since symptom onset (median, 30 days) and (D) > 180 days median post symptom onset (median, 203 days). Percentages of cytokine-expressing CD4+ T cells are background subtracted and only subsets with detectable T cells are displayed. Data shown were restricted to positive responders and a single data point per individual per time frame. All subsets were also evaluated for expression of IL-4, IL-5, IL-13, IL-17, and perforin and were found to be negative.

(E) Bar graphs indicate the proportion of COVID-19 convalescent patients who had a positive CD4+ T cell response to the individual SARS-CoV-2. peptide pool *ex vivo* stimulations. Some antigens were combined for stimulation as indicated.

(F) For each subject with positive SARS-CoV-2-specific CD4+ T cells, the proportion of the total SARS-CoV-2 responding CD4+ T cells that are specific for each stimulation.

we assessed T cell responses to the SARS-CoV-2 structural (S, E, M, and N) and accessory proteins (ORF 3a, 6, 7a, 7b, and 8) using overlapping peptide pools that span the sequences of these proteins.

Among COVID-19 patients, 89% (102/113) mounted CD4+ T cell responses (Figure 5A) recognizing at least one SARS-CoV-2 structural protein that was detectable at one or more visits. By contrast, SARS-CoV-2 specific CD4+ T cells were

rarely detected in the uninfected control group using this assay (Figure S3C). Antigen-specific CD4+ T cells expanded over the first month after infection and then gradually declined over subsequent months. Their estimated half-life was 207 days (95% CI [104, 211]) as shown in Figure 5A, and these findings are supported by the individual CD4+ T cell response levels and slopes after day 30 (slope = −0.0033, 95% CI [-0.0017, −0.0066], $p < 0.0001$) (Figures S4C and S4D). Of note, we observed a wide range in the total magnitude of responses, some reaching >1% of circulating CD4+ T cells, and an overall median frequency of 0.51% (Figures 5A and S5).

To better characterize the development of T cell memory in SARS-CoV-2 infection, we examined the differentiation profiles of virus-specific T cells longitudinally in COVID-19 patients. Based on CD45RA and CCR7 expression, SARS-CoV-2-specific CD4+ T cells were primarily central memory phenotype (CD 45RA- CCR7+) and to a lesser extent effector memory (CCRA-CCR7-); this profile of the memory T cell subsets was very consistent between subjects and stable over time (Figure 5B). The antigen-specific CD4+ T cells were Th1-biased with a predominant CXCR3+CCR6- phenotype, and highly polyfunctional, with simultaneous detection of antigen-specific CD154, IFN-γ, IL-2, TNF-α and less frequently granzyme B in the early expansion phase (21–60 days post symptom onset; median, 30 days) (Figure 5C). Interestingly, many of the virus-specific CD4+ T cells also exhibited this polyfunctionality at the memory time point (>180 days post symptom onset; median, 203 days) (Figure 5D). Circulating SARS-CoV-2-specific Th2 (IL-4, IL-5, and IL-13), Th17 (IL-17), or perforin-expressing subsets were not detected (Figures 5C and 5D).

Next, we examined the CD8+ T cell responses in COVID-19 patients and found that 69% generated CD8+ T cells recognizing at least one SARS-CoV-2 structural protein that were detectable at one or more visits (Figure 6A), in contrast to infrequent to rare, low-level antigen-specific responses in the uninfected control donors (Figure S3D). Expansion of CD8+ T cells occurred over the first month and then frequencies gradually declined, with a half-life of 196 days (95% CI [92, 417]) and a negative estimated slope after 30 days of symptom onset (slope = −0.004, 95% CI [-0.002, −0.008], $p < 0.0001$) (Figure 6A). The median frequency of SARS-CoV-2-specific CD8+ T cells was 0.2%, indicating a lower overall response magnitude than observed for CD4+ T cells. However, like the CD4+ T cells, a wide range in magnitudes was observed with many SARS-CoV-2-specific CD8+ T cell frequencies above 1% and even up to 12% (Figure 6A).

A very different pattern of phenotypic changes were observed with virus-specific CD8+ T cells compared to what we saw with the CD4+ T cells (Figure 6B versus Figure 5B). In contrast to the dominance of the central memory subset with SARS-CoV-2-specific CD4+ T cells, the vast majority of the virus-specific CD8+ T cells showed an effector memory phenotype during the early phase of the response. However, this population of SARS-CoV-2-specific effector memory (CD45RA-CCR7-) contracted over time (slope = −0.904, $p < 0.0001$; Figure 6B) and simultaneously there was an increase in the proportion of the TEMRA (CD45RA+CCR7-) subset of virus-specific CD8+ T cells (slope = 0.075, $p < 0.0001$; Figure 6B). A small but stable fraction of SARS-CoV-2-specific CD8+ T cells expressed a central memory phenotype (slope = 0.024, p = ns; Figure 6B).

The SARS-CoV-2-specific CD8+ T cells were highly polyfunctional with the highest magnitude populations secreting IFN-γ, TNF-α, and granzyme B; other dominant subsets also expressed IL-2 or perforin (Figures 6C and 6D). This polyfunctional profile was seen in the expansion phase (median 30 days; Figure 6C) and also at the later time points (>180 days post symptom onset; median 203 days; Figure 6D). It is important to note that this pattern of CD8+ T cell differentiation has been described in detail after vaccination in humans with the live attenuated yellow fever virus vaccine (YFV-17D).[15] This YFV-17D vaccine generates long-lived and functional virus-specific memory CD8+ T cells that persist in humans for decades.[15,16] That the CD8+ T cell differentiation program after COVID-19 infection resembles what is seen after YFV infection of human suggests that COVID-19 patients may also generate long-lived CD8+ T cell memory.

CD4+ and CD8+ cells target different SARS-CoV-2 antigen specificities

The majority of COVID-19 patients generated CD4+ T cells that recognized most SARS-CoV-2 viral structural and accessory proteins, with the highest percentage responding to S2 (78%) and S1 (69%) (Figures 5E and 5F). Among the COVID-19 subjects with positive responses, the proportion of SARS-CoV-2-specific CD4+ T cells reacting to each peptide pool was evenly distributed (Figure 5F). Thus, CD4+ T cells equally targeted multiple SARS-CoV-2 proteins.

In contrast to the results seen with CD4+ T cells, SARS-CoV-2-specific CD8+ T cells showed preferential recognition of the nucleocapsid protein. The dominant CD8+ T cell response rate was directed to the nucleocapsid (57%); followed by ORFs 7a, 7b, and/or 8 (25%); S1 (25%); ORFs 3a and/or 6 (16%); S2 (12%); and E and/or M (9%) (Figure 6E). Also, among the COVID-19 patients with CD8+ T cell responses, there was a bias with the largest percentage (median, 43%) reacting to the nucleoprocapsid protein (Figure 6F). While SARS-CoV-2 CD8+ T cell responses rates were much lower in uninfected controls, when present in a few control donors with lower frequencies, these were also targeted to the nucleocapsid protein (Figure S3D). A likely explanation for these findings is that in SARS-CoV-2 infection, antigen-presenting cells *in vivo* may display a higher proportion of peptides derived from the nucleocapsid protein and hence more nucleocapsid-specific CD8+ T cells are generated during infection. This has interesting implications suggesting that nucleocapsid-specific CD8+ T cells might be more efficient in recognizing virally infected cells.

Age and disease severity are significantly associated with magnitude of SARS-CoV-2 immune responses

We evaluated whether COVID-19 patient age, disease severity, or gender could account in part for the heterogeneity observed among the SARS-CoV-2-specific immune responses as estimated from the individual models (post day 30 for cellular and post day 42 for antibody responses). We observed that age was significantly associated with higher immune responses to SARS-CoV-2, independently of any covariation with disease

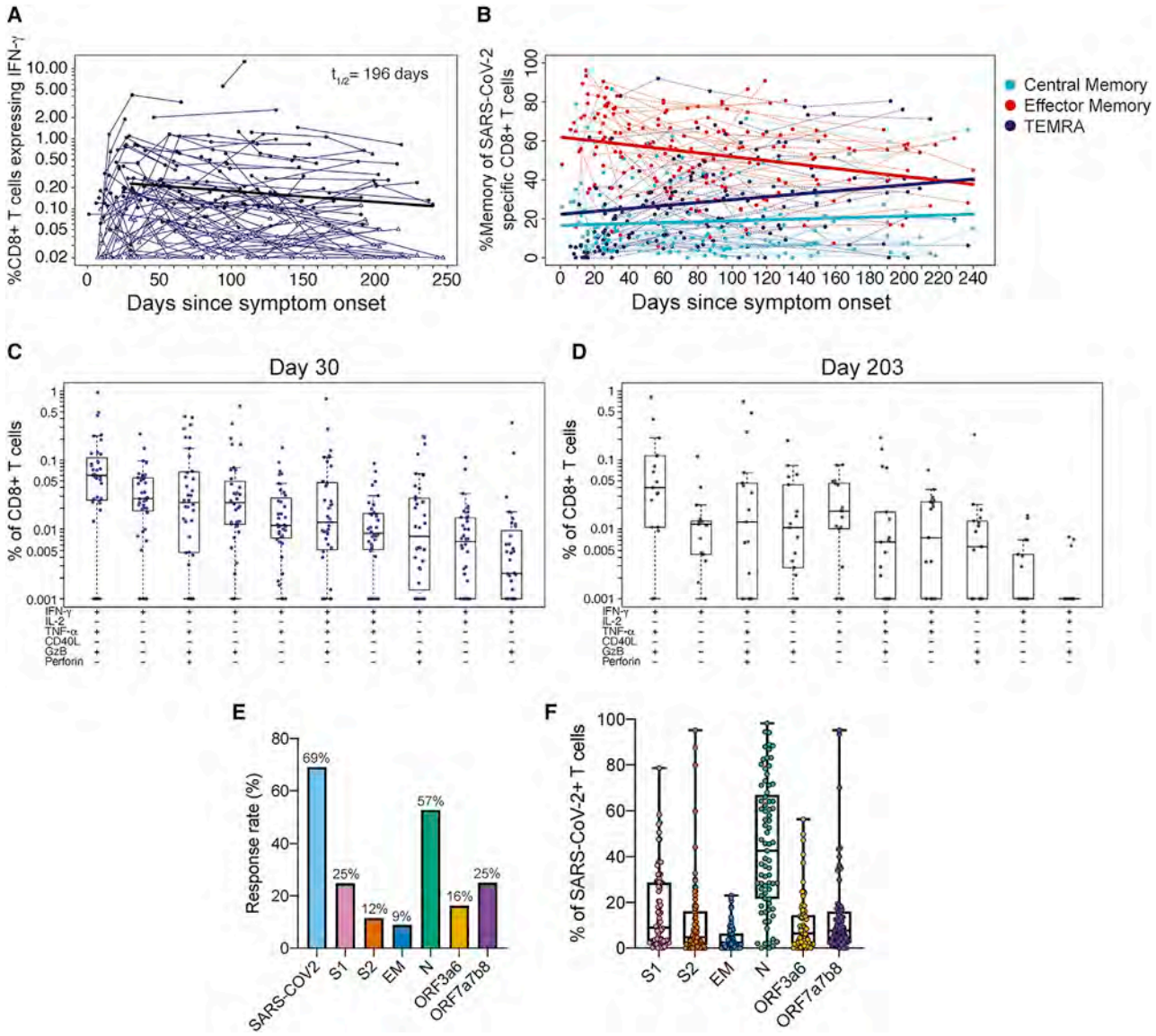

Figure 6. CD8+ T cell responses to SARS-COV-2 antigens

(A) The sum of background-subtracted CD8+ T cells expressing IFN-γ (with or without other cytokines), in response to peptide pools covering SARS-CoV-2 structural proteins: S1, S2, envelope (E), membrane (M), nucleocapsid (N), and the following ORFs: 3a, 3b, 6, 7a, 7b, and 8 (n = 114; tested in singlets) for each individual/time point. Each sample that is positive (MIMOSA) for at least 1 SARS-CoV-2 antigen is indicated by a solid circle, whereas samples that are negative for all of the SARS-CoV-2 antigens at that time point are indicated by open triangles. The bold black line represents the median fitted curve from a nonlinear mixed effects model of post-day 30 responses among those with a positive response to the antigen(s) under consideration at [3]1 time point; $t_{1/2}$ shown is the median half-life estimated from the median slope, with 95% CI [92, 417].

(B) The proportion of SARS-CoV-2-specific CD8+ T cells by memory phenotype over time: effector memory (EM; CCR7- CD45RA-), T_{EMRA} (CCR7- CD45RA+), and central memory (CM; CCR7+ CD45RA-). Analyses were restricted to positive responders.

(C and D) Polyfunctionality of SARS-CoV-2-specific CD8 T cells at (C) 21–60 days post symptom onset (median, 30 days) and (D) >180 days median post symptom onset (median, 203 days). Percentages of cytokine expressing CD8+ T cells are background subtracted and only subsets with detectable T cells are displayed. Data shown were restricted to positive responders and a single data point per individual per time frame. All CD8+ T cell subsets were also evaluated for expression of IL-4, IL-5, IL-13, and IL-17 and were found to be negative.

(E) The bar graphs indicate the proportion of COVID-19 convalescent patients who had a positive CD8+ T cell response to the individual SARS-CoV-2 stimulations.

(F) The fraction of the total SARS-CoV-2 responding CD8+ T cells per subject that are specific for each peptide pool.

severity (Figure 7A). Neutralizing antibody titers and IgG antibody responses to nucleocapsid increased 1.35-fold and 1.25-fold, respectively, with each decade of age and the same disease severity (95% *Cis* [1.19, 1.54] and [1.08, 1.43], p values < 0.003). Similarly, increased age positively correlated with increased frequencies of spike and RBD-specific IgG+ memory

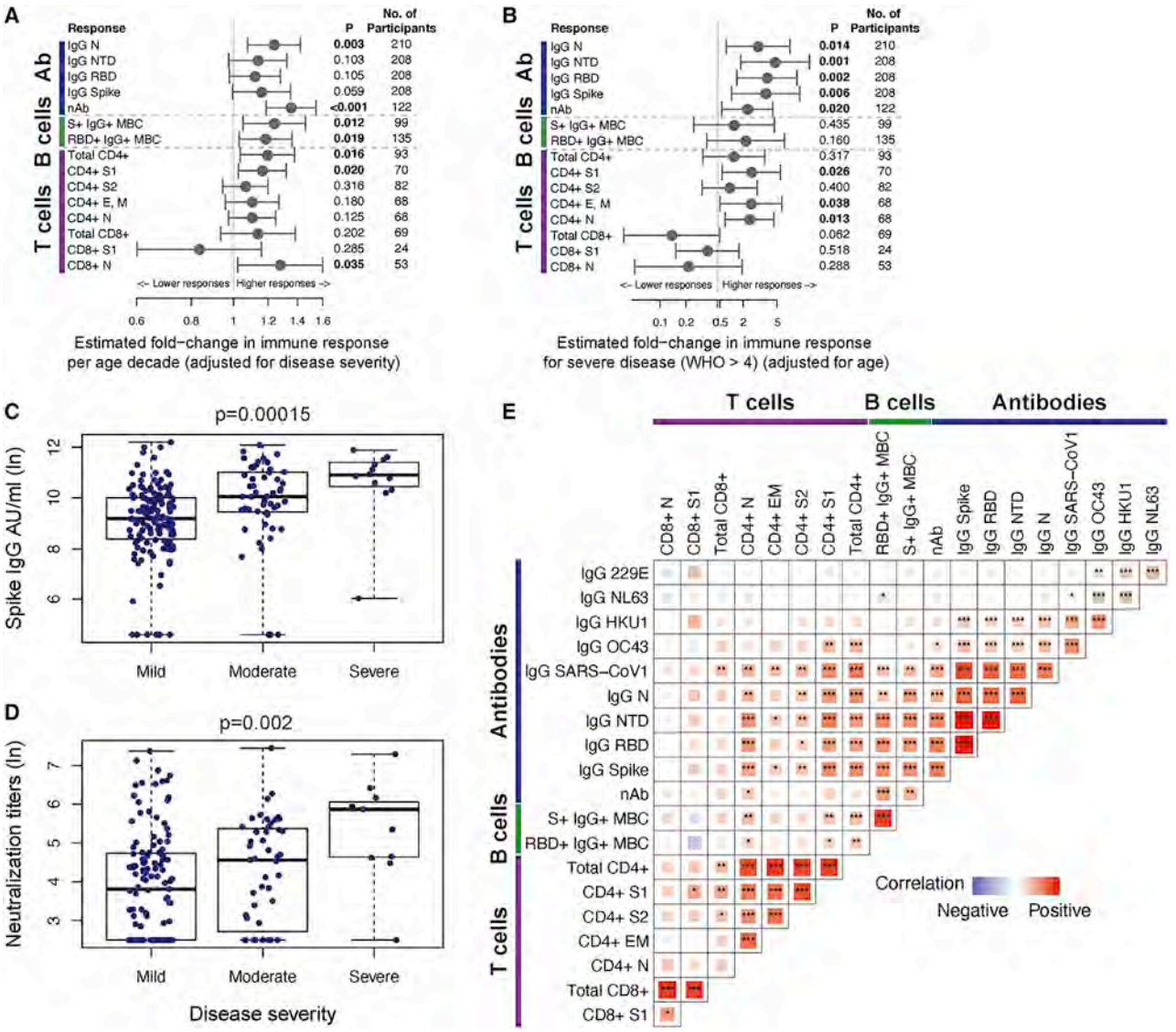

Figure 7. Correlations between SARS-CoV-2-specific immune responses and assessment of covariates

(A) The forest plot depicts the estimated fold-change in the level of each immune response per decade of age, with 95% Wald-based CIs and p values.

(B) The forest plot shows the estimated fold-change in the level of each immune response for severe (WHO score >4) versus non-severe (WHO score £4) disease, with 95% Wald-based CIs and p values. S1 CD8+ T cell responses compared moderate-severe (WHO score >2) to mild (WHO score £2) disease as there were no participants with severe disease with at least one positive S1 CD8+ T cell response post-day 30. Estimates in (A) and (B) are from mixed effects models of post-day 30 (B and T cell responses) or post-day 42 (antibody responses) among responders that account for fixed effects of age and disease severity on the level of immune response.

(C and D) Univariate assessment of disease severity on the magnitude of (C) spike IgG antibodies and (D) SARS-CoV-2 neutralizing antibodies at day 120 is shown for mild (WHO score: 0-2), moderate (WHO score: 3-4), and severe disease (WHO score: 5+); p values from one-way ANOVA.

(E) The heatmap shows Spearman correlations between critical SARS-CoV-2 memory immune responses (day 30 B and T cell responses and day 180 antibody responses) with significance levels: *p < 0.05, **p < 0.01, and ***p < 0.001. The tile size and color intensity correspond to the absolute value of the Spearman rank correlation coefficient, with red or blue indicating a positive or negative correlation, respectively. Day 30, 42, and 180 immune responses were estimated from mixed effects models of the longitudinal SARS-CoV-2 binding antibodies, SARS-CoV-2 neutralizing antibodies, CD4+ and CD8+ T cell responses, and B cell responses.

B cells, with 1.19- to 1.24-fold higher responses per decade of age (p values < 0.02; Figure 7A), accounting for disease severity. Increased age also correlated with higher SARS-CoV-2 and S1-specific CD4+ T cell responses (1.16- to 1.20-fold increase by decade of age, p values < 0.02) and N-specific CD8+ T cell responses (1.24-fold increase by decade of age, p = 0.039) accounting for disease severity (Figure 7A).

Since the cohort included primarily persons with mild-to-moderate COVID-19, we had limited ability to assess the relationship of severe disease and SARS-CoV-2 immune responses,

especially among the cellular responses. However, we found that after accounting for age, severe disease (WHO score >4) was associated with higher IgG antibodies to nucleocapsid, spike, RBD, and NTD (Figures 7B and 7C), and SARS-CoV-2 neutralization titers (Figure 7D). Severe disease was also associated with 2.30- to 2.46-fold higher S1, E and/or M, and nucleocapsid-specific CD4+ T cells (all p values < 0.05; Figure 7B). We found no significant relationships between gender and the immune responses evaluated, apart from 1.66-fold higher IgG NTD responses antibodies among males compared to females, after accounting for age and disease severity (95% CI [1.08, 2.55], p = 0.022). In all, our analyses suggest that there are synergistic but also independent mechanisms driving higher adaptive immune responses in COVID-19 patients who are older and/or who experienced more severe disease.

Early SARS-CoV-2 B and T cell responses correlated with durable spike and RBD IgG antibody binding and neutralization titers

We assessed correlations between SARS-CoV-2-specific immune responses using the individual-level models to interpolate the magnitude of responses for each COVID-19 patient at early (day 30) or later (day 180) convalescent time points (Figure 7E). We found that durable serum neutralization titers correlated with the magnitude of IgG+ binding antibodies to spike, NTD and RBD at day 180 each (day 180; Spearman R = 0.62, 0.61, and 0.61, respectively; all p values < 0.0001). Similarly, the frequency of RBD+ IgG+ memory B cells at day 30 correlated with the maintenance of RBD+ IgG antibodies (day 180; Spearman R = 0.53, p < 0.0001) and neutralization antibody titers (day 180; Spearman R = 0.48, p < 0.0001). We also observed that the magnitude of S1-specific CD4+ T cells at day 30 correlated with durable IgG antibodies against spike (day 180; Spearman R = 0.56, p < 0.0001), NTD (Spearman R = 0.62, p < 0.0001), and RBD (Spearman R = 0.47, p = 0.0002) (Figure 7E). These findings are consistent with early SARS-CoV-2 memory B cells and CD4+ T cells supporting the generation of durable antibody responses.

DISCUSSION

Establishing immune memory is essential in the defense against SARS-CoV-2 infection. To end the COVID-19 pandemic, it is critical to know how long immunity against SARS-CoV-2 will persist after infection and whether it will be sufficient to prevent new infections and severe disease in years to come. Identifying, in-depth, the adaptive immune components leading to recovery and modeling the trends of each response was enabled by the longitudinal sampling of a large number of COVID-19 patients. Here, we show that most convalescent COVID-19 patients mount durable antibodies, B cells, and T cells specific for SARS-CoV-2 up to 250 days, and the kinetics of these responses provide an early indication for a favorable course ahead to achieve long-lived immunity. Because the cohort will be followed for 2–3 more years, we can build on these results to define the progression to long-lived immunity against this novel human coronavirus, which can guide rational responses when future outbreaks occur.

The hallmark of the initial immune defense against SARS-CoV-2 is the emergence of antibodies recognizing the SARS-CoV-2 spike protein, including the RBD and NTD components of the S1 subunit, during the early phase of viral replication. These antibodies are likely secreted from plasmablasts rapidly generated from B cells that are activated upon their first encounter with the pathogen spike antigen. The brisk rise over the first month of infection, followed by a fast decline of the circulating spike IgG and IgA antibodies, is a consistent finding and likely explained by the disappearance of the short-lived plasmablasts. These events occur even sooner for the spike IgM and nucleocapsid antibodies.

Some antibodies that bind to specific epitopes on the spike RBD and NTD can block SARS-CoV-2 infection of respiratory epithelial cells by inhibiting the interactions of the viral spike with the ACE2 receptor.[17-20] Thus, as expected, the early rise and decline of antibodies neutralizing live SARS-CoV-2 were similar to the kinetics of antibodies binding the spike and RBD protein. The striking finding is the bi-phasic curve of the spike-specific binding and neutralizing antibody responses when analyzed with the power law model, which provides a better fit for the antibody kinetics after the peak response.[21] This bi-phasic decline accords with other recently published observations on SARS-CoV-2 serological kinetics.[22,23] With sampling data extended to 250 days, we were able to detect a slowing of the decay of these functional antibodies toward a plateau level, suggestive of the generation of longer-lived plasma cells, and durable antibody responses. The importance of these observations is that following recovery, neutralizing antibodies may persist, albeit at low levels, and may act as the first line of defense against future encounters of SARS-CoV-2 and possibly related human coronaviruses.

Another interesting finding of this investigation is the remarkably stable antibody responses among the pre-pandemic and COVID-19 patients to the common human coronaviruses that are acquired in children and adults. These data are most consistent with the generation of long-lived plasma cells and refute the current notion that these antibody responses to human coronaviruses are short lived. Moreover, the COVID-19 patients mounted increased IgG antibody responses to SARS-CoV-1, a related pathogen that none likely had experienced previous exposure to. This finding is consistent with the booster response of SARS-CoV-1 neutralizing antibodies that we recently observed following SARS-CoV-2 mRNA vaccination.[3,24] Taken together, these results may have implications for a broader strategy for vaccines targeting multiple betacoronaviruses.

The durable antibody responses in the COVID-19 recovery period are further substantiated by the ongoing rise in both the spike and RBD memory B cell responses after over 3–5 months before entering a plateau phase over 6–8 months. Persistence of RBD memory B cells has been noted.[25-27] We presume this may be explained by sustained production of memory B cells in germinal centers of lymph nodes draining the respiratory tract in the early months, followed by the memory B cell redistribution into the circulation as the germinal centers begin to recede. Thus, the induction and maintenance of memory B cells and, over time, long-lived plasma cells, will continue to furnish higher affinity antibodies if re-exposures occur.

In contrast to spike memory B cell kinetics, SARS-CoV-2-specific CD4+ and CD8+ memory T cells each peak early, within the first month, but then slowly decline over the next 6–7 months. Central memory Th1-type CD4+ T cells dominate throughout the early infection and recovery period. However, the CD8+ T cells exhibit a predominant effector memory phenotype early that transitions to those effector memory cells re-expressing CD45RA, maintaining expression of antiviral cytokines and effector functions that have been shown to provide protective immunity against other viral pathogens. We also provide clear evidence that the CD4+ T cells mount a broader antigen-specific response across the structural and accessory gene products, whereas the CD8+ T cells are predominantly nucleocapsid specific and spike-specific responses are substantially lower in frequency.

Our study demonstrates the considerable immune heterogeneity in the generation of potentially protective response against SARS-CoV-2, and by focusing on the dynamics and maintenance of B and T cell memory responses, we were able to identify features of these early cellular responses that can forecast the durability of a potentially effective antibody response. The ability to mount higher frequencies of RBD-specific memory IgG+ B cells early in infection was the best indicator for a durable RBD-specific IgG antibody and neutralizing antibody response. In addition, higher frequency CD4+ T cells were associated with stronger spike IgG and neutralizing antibody responses. However, the induction and peak response of SARS-CoV-2-specific CD8+ T cells occurs independently to these antibody responses. Interestingly, while it has been widely reported that age correlates with COVID-19 disease severity, we found that age and disease severity were independent co-variates associated with the magnitude of both SARS-CoV-2-specific CD4+ T cell and humoral SARS-CoV-2 immunity, but not with the magnitude of CD8+ T cell responses. In the case of T cells, whether the T cell differences are related to the frequencies or specificities of pre-existing coronavirus CD4+ and CD8+ T cell immunity will require additional future analysis.

The COVID-19 pandemic remains a global public health threat after 1 year of overwhelming disruption and loss. Overcoming the challenges to end the pandemic is accentuated by the recognition that SARS-CoV-2 can undergo rapid antigenic variation that may lower vaccine effectiveness in preventing new cases and progression to severe disease.[24,28,29] Our findings show that most COVID-19 patients induce a wide-ranging immune defense against SARS-CoV-2 infection, encompassing antibodies and memory B cells recognizing both the RBD and other regions of the spike, broadly-specific and polyfunctional CD4+ T cells, and polyfunctional CD8+ T cells. The immune response to natural infection is likely to provide some degree of protective immunity even against SARS-CoV-2 variants because the CD4+ and CD8+ T cell epitopes will likely be conserved. Thus, vaccine induction of CD8+ T cells to more conserved antigens such as the nucleocapsid, rather than just to SARS-CoV-2 spike antigens, may add benefit to more rapid containment of infection as SARS-CoV-2 variants overtake the prevailing strains.

Limitations of the study

Our study evaluates COVID-19 patients only up to 8 months and requires models to estimate immune response half-lives thereafter. Because our longitudinal study will extend beyond 2 years, we can corroborate our models with subsequent experimental data on the persistence of immune memory. Our study population was primarily outpatients with mild-to-moderate COVID-19 and thus we were unable to evaluate immune memory in those with the extreme presentations, both asymptomatic and severe COVID-19. However, mild-moderate illness accounts for >80% of COVID-19 cases[30], highlighting the relevance of our findings over time.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2021.100354.

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AUTHOR CONTRIBUTIONS

M.J.M and R. Ahmed conceived the study. M.J.M., S.E., J.C., E.J.A., A.K.M, N.R., and J.O.K. established the cohort and recruited the participants. S.L.L., M.P.L., C.W.D., M.P.G., S.G., K.A.S., G.M., C.N., V.V.E., L.L., and D.S.S. conducted serological assays and related analyses. H.A., V.I.Z., B.P., and Z.M. conducted formal statistical analyses and modeling. K.W.C., R.W., and L.E.N. planned, performed, and analyzed antigen-specific B cell flow cytometry. S.C.D., K.W.C., and S.F. conceived, supervised, performed, and analyzed T cell experiments. V.E.E, K.F., and L.L. performed FRNT assays. K.W.C., S.L.L., and Z.M. drafted the original manuscript; M.J.M., M.S.S., and R. Ahmed edited the manuscript. All authors read and approved the manuscript. M.J.M., R.A, J.W., and M.S.S. secured funds and supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Anti-Human CD3/BV510	BD Biosciences	564713; RRID:AB_2738909
Mouse Anti-Human CD14/BV510	BD Biosciences	563079; RRID:AB_2737993
Mouse Anti-Human CD56/BV510	BD Biosciences	563041; RRID:AB_2732786
Mouse Anti-Human CD19/BUV395	BD Biosciences	563549; RRID:AB_2738272
Mouse Anti-Human CD20/BUV737	BD Biosciences	612849; RRID:AB_2870169
Mouse Anti-Human CD21/PE-Cy7	BD Biosciences	561374; RRID:AB_10681717
Mouse Anti-Human CD27/BV605	BD Biosciences	302830; RRID: AB_2561450
Mouse Anti-Human CD38/BB700	BioLegend	566445; RRID:AB_2744375
Mouse Anti-Human IgA/VioBlue	Miltenyi Biotec	130-114-005; RRID:AB_2733958
Mouse Anti-Human IgD/BV650	BD Biosciences	740594; RRID:AB_2740295
Mouse Anti-Human IgG/BV786	BD Biosciences	564230; RRID:AB_2738684
Mouse Anti-Human IgM/PE-Dazzle 594	BioLegend	314530; RRID:AB_2566483
Streptavidin (PE)	Invitrogen	S21388; RRID:AB_2892541
Streptavidin (AF488)	Invitrogen	S32354; RRID:AB_2315383
Streptavidin (AF647)	Invitrogen	S32357; RRID:AB_2892542
Live/Dead Fixable Aqua Stain	Invitrogen	L34957
Fixable Viability Dye/eFluor 450	Invitrogen	65-0863
Mouse Anti-Human CD14/BUV661	BD Biosciences	741684; RRID:AB_2868407
Mouse Anti-Human CD19/BUV563	BD Biosciences	612916; RRID:AB_2870201
Mouse Anti-Human CD16/BV570	BioLegend	302036; RRID:AB_2632790
Mouse Anti-Human CD56/BV750	BioLegend	362556; RRID:AB_2801001
Mouse Anti-Human CD3/APC-Fire750	BioLegend	300470; RRID:AB_2629689
Mouse Anti-Human CD4/BV480	BD Biosciences	566104; RRID:AB_2739506
Mouse Anti-Human CD8/BUV805	BD Biosciences	612889; RRID:AB_2833078
Mouse Anti-Human CD197(CCR7)/BV605	BioLegend	353224; RRID:AB_2561753
Mouse Anti-Human CD45RA/BUV496	BD Biosciences	750258; RRID:AB_2874456
Mouse Anti-Human CD25/BV650	BD Biosciences	563719; RRID: AB2744337
Rat Anti-Human FOXP3/PE-Cy5.5	Invitrogen	35-4776-42; RRID:AB_11218682
Mouse Anti-Human CD32/PE-Dazzle	BioLegend	303218; RRID:AB_2716072
Mouse Anti-Human CD65/BV711	BioLegend	305042; RRID:AB_2800778
Mouse Anti-Human CD183/PE-Cy5	BD Biosciences	551128; RRID:AB_394061
Mouse Anti-Human CD196 (CCR6)/BV786	BD Biosciences	563704; RRID:AB_2738381
Rat Anti-Human CD294 (CRTH2)/PE	BioLegend	350106; RRID:AB_10900060
Mouse Anti-Human IFN-g/V450	BD Biosciences	560371; RRID:AB_1645594
Rat Anti-Human IL-2/APC	BioLegend	500310; RRID:AB_315097
Mouse Anti-Human TNF/BUV395	BD Biosciences	563996; RRID:AB_2738533
Mouse Anti-Human IL-17A/PE-Cy7	BioLegend	512315; RRID:AB_2295923
Rat Anti-Human IL-4/BB700	BD Biosciences	Custom
Rat Anti-Human/Anti-Mouse IL-5/BB630	BD Biosciences	Custom
Rat Anti-Human IL-13/BV421	BD Biosciences	Custom
Mouse Anti-Human CD154 (BUV737)	BD Biosciences	748983; RRID:AB_2873383
Mouse Anti-Human Granzyme B/AF700	BD Biosciences	560213; RRID:AB_1645453
Mouse Anti-Human Perforin/FITC	BD Biosciences	353310; RRID:AB_2571967
Mouse Anti-Human Ki-67/BB660	BD Biosciences	Custom

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
icSARS-CoV-2-mNG	Xie et a.	N/A
Chemicals, peptides, and recombinant proteins		
SARS-CoV-2 Spike peptides	Biosynthesis	Custom
SARS-CoV-2 E, M, N and ORF peptides	Genscript	Custom
SARS-CoV-2 Spike protein (S6P)	Fred Hutchinson Cancer Research Center	Custom
SARS-CoV-2 RBD protein	Fred Hutchinson Cancer Research Center	Custom
Methylcellulose	Sigma-Aldrich	M0512-250G
TrueBlue Peroxidase Substrate	KPL	5510-0050
Critical commercial assays		
V-PLEX COVID-19 Coronavirus Panel 2 (IgG) Kit	Meso Scale Discovery	K15369U
V-PLEX COVID-19 Coronavirus Panel 2 (IgA) Kit	Meso Scale Discovery	K15371U
V-PLEX COVID-19 Coronavirus Panel 2 (IgM) Kit	Meso Scale Discovery	K15370U
Experimental models: Cell lines		
VeroE6 C1008 cells	ATCC	Cat# CRL-1586; RRID:CVCL_0574
Software and algorithms		
FlowJo	BD Biosciences	V9.9.4
R	R Foundation for Statistical Computing	V3.6.1
GraphPad Prism	GraphPad	V7, 8 and 9
Viridot	Katzelnick et al.	https://github.com/leahkatzelnick/Viridot
Monolix	Lixoft	MonolixSuite2019R1
Other		
ELISPOT reader	Immunospot	CTL ImmunoSpot S6 Universal Analyzer

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, M. Juliana McElrath (jmcelrat@fredhutch.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The underlying data for this paper will be shared by the lead contact upon request without restriction.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study populations

Two longitudinal COVID-19 cohort studies at Fred Hutchinson Cancer Research Center (Seattle, Washington) and Emory University (Atlanta, Georgia) began after receiving institutional review board approvals (IRB 10440, IRB 00001080 and IRB00022371). Adults ^{3}18 years were enrolled who met eligibility criteria for SARS-CoV-2 infection and provided informed consent. Study participants provided medical history of co-morbidities, presentation of SARS-CoV-2 infection onset and disease course, and peripheral blood at initial and follow up visits for analysis of serum antibody and cellular immune responses. Additional longitudinal archived sera and PBMC from pre-pandemic study populations from Emory and Seattle served as controls for the immune assays.

The Atlanta study population included adult volunteers over the age of 18 who were diagnosed with COVID-19 by a commercially available SARS CoV-2 PCR assay, rapid antigen test, or clinical syndrome only (later confirmed with serology) due to limited SARS-CoV-2 testing during the early period of the pandemic. Ambulatory participants were recruited through local advertisements,

internet-based avenues (such as social media, listserves), COVID-19 testing sites, and primary care clinics. Hospitalized patients were identified through SARS-CoV-2 testing. Informed consent was obtained from all participants prior to conduct of study procedures. Initial acute peripheral blood samples were collected from hospitalized patients at the time of enrollment. Convalescent samples from hospitalized patients were collected when the patients were able to return for a visit to the clinical research site at the next study visit. Serial peripheral blood samples were collected starting at about 30 days after the onset of COVID-19 symptoms and/or after PCR positivity for SARS-CoV-2. Thereafter, samples were collected at 3, 6, and 9 months. The study is ongoing with expected completion of sample collection from participants in February 2023. Participants were excluded if they were immunocompromised, HIV positive, had active hepatitis B or C virus infection, used immunosuppressive drugs for 2 weeks or more in the preceding 3 months, received blood products or immune globulin 42 days prior to enrollment, received convalescent COVID-19 plasma, or were pregnant or breast feeding. We report on 110 participants to date, of which 73% were diagnosed by SARS-CoV-2 PCR, the remaining were diagnosed by rapid antigen test or serology. Demographic features of the participants are as follows: median age was 48; 45% were male; the majority (80%) were white, 11% Black/African American, 6% Asian, and 8% were Hispanic/Latinx ethnicity. The most frequent co-morbid conditions were hypertension, obesity, heart disease and diabetes mellitus. The most frequent COVID-19 symptoms were myalgia/fatigue, fever, cough, headache, loss of smell and taste (Table S1). Hospitalized patients were older, with a median age of 56; a higher percentage were Black/African American (27%); and 100% had fever.

Longitudinal pre-pandemic sera samples from Emory were collected from individuals participating in a yellow fever vaccine study from 2014-2016 or an influenza vaccine study from 2015-2018[15,31]. Data were included for analysis of binding antibody responses and are presented as days post-irrelevant (yellow fever) vaccination. The study was approved by the Emory University IRB and donors were enrolled after providing written informed consent.

The Seattle COVID-19 Cohort study participants were recruited from the Seattle metropolitan area by social media advertisements, partnership with the local emergency medical service and by word of mouth. Study participants were screened and enrolled by the Seattle Vaccine Trials Unit staff. Eligibility criteria included adults at risk for SARS-CoV-2 infection or those diagnosed with COVID-19 by a commercially available SARS-CoV-2 PCR assay or blood antibody test and willing to have at least four blood draws collected over one year. Exclusion criteria included pregnancy and inability to donate blood.

Informed electronic consent was obtained from all Seattle participants during a screening phone call with study clinical staff. Interested participants were screened, consented and medical history and COVID-19 illness onset date and symptoms collected. Participants undiagnosed with COVID-19 had a nasopharyngeal (NP) swab collected and tested for SARS-CoV-2 via an FDA-approved PCR test and blood was collected for SARS-CoV-2 antibody (Abbott) and study assays. Those with either a positive PCR or antibody test were asked to return for future blood draws. Those who tested negative were asked to return as controls for the positive cohort and in case they tested positive in the future. Participants with a positive test prior to study enrollment or those diagnosed in study were asked to provide blood donation at approximately 7 days, 2 weeks, 1, 2, 3, 4, 6, 9- and 12-months post symptom onset. After completing one year of study, participants will be given the option of continuing the longitudinal study for up to two or more years. At each study visit, participant symptoms and medical history is updated. Those with COVID-19 symptoms after enrollment in all groups are offered a nasopharyngeal swab PCR SARS-CoV-2 test.

As of October 2020, 805 individuals have contacted the Seattle COVID-19 cohort study and 425 have enrolled. This includes 281 negative and 144 SARS-CoV-2 positive participants. Reasons for not enrolling include lack of interest, not meeting the eligibility criteria, inability to travel to blood draw location and inability to collect study blood. No participants have terminated from the study. Study enrollment and follow-up remains ongoing. Samples from SARS-CoV-2 negative subjects were included in B and T cell assays as 'contemporaneous' negative controls.

Peripheral blood mononuclear cells (PBMC) were obtained from HIV-1 seronegative donors who were recruited at the Seattle Vaccine Trials Unit before 2019 as part of the study "Establishing Immunologic Assays for Determining HIV-1 Prevention and Control." All participants signed informed consent, and the Fred Hutchinson Cancer Research Center IRB (Seattle, WA, USA) institutional human subjects review committee approved the protocol prior to study initiation. Pre-pandemic samples from this cohort were used as assay controls in B and T cell assays.

METHOD DETAILS

PBMC processing

PBMC for cellular assays were isolated by density centrifugation and cryopreserved from ACD-anticoagulated whole blood within eight h of venipuncture, as described previously [32]. Sera were also processed and cryopreserved within 4 h after collection.

Antibody binding assay

Antibody binding titers were measured using a multiplex plate coated with the SARS-CoV-2 spike, SARS-CoV-2 spike receptor binding domain, SARS-CoV-2 spike N-terminal domain, SARS-CoV-2 nucleocapsid, SARS-CoV-1 spike, 229E spike, NL63 spike, HKU1 spike, and OC43 spike proteins (Mesoscale Discovery). Plates were blocked with 150ml/well with 5% bovine serum albumin in phosphate buffered saline (PBS) and shaken at 700 RPM at room temperature for at least 30 min. Plates were washed 3 times with 150ml/well 0.05% Tween-20 in PBS. Serum and plasma samples were added to the plate at dilutions between 1:500 and 1:50,000 and shaken at 700 RPM at room temperature for 2 h. Following a wash, plates were incubated with 50ul/well of Sulfo-Tag anti-human

IgG, IgA, or IgM detection antibody and shaken at 700RPM at room temperature for 1 h. After a subsequent wash, 150ml/well of MSD GOLD read buffer was added to the plate and plates were immediately read on the MSD instrument to measure light intensity. Antibody levels are reported as arbitrary units/mL (AU/mL) based on normalization to a standard curve.

Viruses and cell lines

VeroE6 cells were obtained from ATCC (clone E6, ATCC, #CRL-1586) and cultured in complete DMEM medium consisting of 1 × DMEM (VWR, #45000-304), 10% FBS, 25mM HEPES Buffer (Corning Cellgro), 2mM L-glutamine, 1mM sodium pyruvate, 1 × Non-essential Amino Acids, and 1 × antibiotics. The infectious clone SARS-CoV-2 (icSARS-CoV-2-mNG), derived from the 2019-nCoV/USA_WA1/2020 strain, was propagated in VeroE6 cells and sequenced [33,34].

Focus reduction neutralization test

Neutralization assays with SARS-CoV-2 virus were performed as previously described [33-35]. Plasma/serum were serially diluted (three-fold) in serum-free Dulbecco's modified Eagle's medium (DMEM) in duplicate wells and incubated with 100–200 FFU infectious clone derived SARS-CoV-2-mNG virus at 37°C for 1 h [33]. The antibody-virus mixture was added to VeroE6 cell (C1008, ATCC, #CRL-1586) monolayers seeded in 96-well blackout plates and incubated at 37°C for 1 h. Post-incubation, the inoculum was removed and replaced with pre-warmed complete DMEM containing 0.85% methylcellulose. Plates were incubated at 37°C for 24 h. After 24 h, methylcellulose overlay was removed, cells were washed twice with PBS and fixed with 2% paraformaldehyde in PBS for 30 min at room temperature. Following fixation, plates were washed twice with PBS and foci were visualized on a fluorescence ELISPOT reader (CTL ImmunoSpot S6 Universal Analyzer) and enumerated using Viridot [36]. The neutralization titers were calculated as follows: 1 - (ratio of the mean number of foci in the presence of sera and foci at the highest dilution of respective sera sample). Each specimen was tested in two independent assays performed at different times. The FRNT-mNG$_{50}$ titers were interpolated using a 4-parameter nonlinear regression in GraphPad Prism 8.4.3. Samples with an FRNT-mNG$_{50}$ value that was below the limit of detection were plotted at 20.

Spike and RBD memory B cell flow cytometry assays

Fluorescent SARS-CoV-2-specific S6P[37] (provided by Roland Strong, Fred Hutchinson Cancer Research Center, Seattle, WA) and RBD (provided by Leonidas Stamatatos, Fred Hutchinson Cancer Research Center, Seattle, WA) probes were made by combining biotinylated protein with fluorescently labeled streptavidin (SA). The S6P probes were made at a ratio of 1:1 molar ratio of trimer to SA. Two S6P probes, one labeled with AlexaFluor488 (Invitrogen), one labeled with AlexaFluor647 (Invitrogen), were used in this panel in order to increase specificity of the detection of SARS-CoV-2-specific B cells. The RBD probe was prepared at a 4:1 molar ratio of RBD monomers to SA, labeled with R-phycoerythrin (Invitrogen). Cryopreserved PBMCs from SARS-CoV-2-convalescent participants and a pre-pandemic SARS-CoV-2-naive donor were thawed at 37°C and stained for SARS-CoV-2-specific memory B cells as described previously[19] with a panel of fluorescently-labeled antibodies (see Key Resource Table). Cells were stained first with the viability stain (Invitrogen) in PBS for 15 min at 4°C. Cells were then washed with 2% FBS/PBS and stained with a cocktail of the three probes for 30 min at 4°C. The probe cocktail was washed off with 2% FBS/PBS and the samples were stained with the remaining antibody panel and incubated for 25 min at 4°C. The cells were washed two times and resuspended in 1% paraformaldehyde/1 × PBS for collection on a LSR II or FACSymphony flow cytometer (BD Biosciences). Data was analyzed in Flow Jo version 9.9.4.

Intracellular cytokine staining (ICS) assay

Flow cytometry was used to examine SARS-CoV-2-specific CD4+ and CD8+ T cell responses using a validated ICS assay. The assay was similar to a published report [5,38,39] and the details of the staining panel are included in the Key Resource Table. Peptide pools covering the structural proteins of SARS-CoV-2 were used for the six-h stimulation. Peptides matching the SARS-CoV-2 spike sequence (316 peptides, plus 4 peptides covering the G614 variant) were synthesized as 15 amino acids long with 11 amino acids overlap and pooled in 2 pools (S1 and S2) for testing (BioSynthesis). All other peptides were 13 amino acids overlapping by 11 amino acids and were synthesized by GenScript. The peptides covering the envelope (E), membrane (M) and nucleocapsid (N) were initially combined into one peptide pool, but the majority of the assays were performed using a separate pool for N and one that combined only E and M. Several of the open reading frame (ORF) peptides were combined into two pools: ORF 3a and 6, and ORF 7a, 7b and 8. All peptide pools were used at a final concentration of 1 mg/mL for each peptide. As a negative control, cells were not stimulated, only the peptide diluent (DMSO) was included. As a positive control, cells were stimulated with a polyclonal stimulant, staphylococcal enterotoxin B (SEB). Cells expressing IFN-g and/or IL-2 and/or CD154 was the primary immunogenicity endpoint for CD4+ T cells and cells expressing IFN-g was the primary immunogenicity endpoint for CD8+ T cells. The overall response to SARS-CoV-2 was defined as the sum of the background-subtracted responses to each of the individual pools. A sample was considered positive for CD4+ or CD8+ T cell responses to SARS-CoV-2 if any of the CD4+ or CD8+ T cell responses to the individual peptide pool stimulations was positive. Positivity was determined using MIMOSA [40]. The total number of CD4+ T cells must have exceeded 10,000 and the total number of CD8+ T cells must have exceeded 5,000 for the assay data to be included in the analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

Binding and neutralizing antibody responses

Mixed effects exponential and power law models were used to analyze waning of antibody (day 42 to day 263 post symptom onset). For binding antibody analyses, antibody (Ab) was natural log transformed, yielding linear equations of the form ln(Ab) = a+b*(day-42) and ln(Ab) = a+b*ln(day/42) for the exponential and power law models, respectively, and fit using the lmer function (lme4 package) in R. Models included population level fixed effects and individual level random effects for intercept and slope and covariance between the random effects. Simplified models – with random effects only for intercept – were also fit. Neutralization antibody data were analyzed in Monolix (Lixoft). For analysis in Monolix, the exponential and power law models were formulated as ordinary differential equations, dAb/dt = k*Ab and dAb/dt = k*Ab/t, respectively, with antibody at day 42 lognormally distributed and lognormal multiplicative error. Neutralization titers < 20 were treated as left censored. For comparison of models, difference in Akaike information criterion (DAIC) > 4 was considered statistically significant. Models (in R and Monolix) were fit using maximum likelihood. To account for repeated-measures, correlations between antibody binding levels and neutralization titers were calculated using a repeated-measures correlation (rmcorr package) in R [41].

B cell responses

We considered linear mixed effects models for B cell response, y_{ij}, as a function of t_{ij}, the j^{th} time since symptom onset for the i^{th} individual, with random effects for intercept and slope and $t_{ij} > 30$ days for all i, j:

$$\log_e y_{ij} = \beta_{0i} + \beta_{1i} t_{ij} + \varepsilon_{ij}$$

where $\beta_{0i} = \beta_0 + b_i$ and $\beta_{1i} = \beta_1 + c_i$ with (b_i, c_i) iid $\sim N_2(0, \Sigma)$, with

$$\Sigma = \begin{bmatrix} \sigma_b^2 & Cov(b,c) \\ Cov(b,c) & \sigma_c^2 \end{bmatrix}$$

and σ_b^2 and σ_c^2 are the between-person variation in the intercept and slope of log B cell responses respectively, *Cov(b, c)* is the covariance between the intercept and slope, and ε_{ij} iid $\sim N(0, \sigma^2)$. The random effects, b_i and c_i, are each assumed to be independent for different individuals and the within-individual errors ε_{ij} are assumed to be independent for different *i, j* and to be independent of the random effects. The function lme from the R package nlme was used to fit the models.

T cell responses

Longitudinal analyses of CD4+ and CD8+ T cell responses were performed for individuals with a positive response for at least one time point 30 days after symptom onset. The MIMOSA (Mixture Models for Single-Cell Assays) [40] model incorporated cell count and cell proportion information to define a positive CD4+/CD8+ T cell response by ICS by comparing peptide pools stimulated cells and unstimulated negative controls. This method assumed a common distribution for cytokine positive CD4+/CD8+ T cells in stimulated and unstimulated samples in non-responders, resulting in paired differences that were zero on average. In contrast, for responders, the distribution of the proportion of cytokine positive cells for stimulated samples was assumed to be greater than for unstimulated samples, resulting in paired differences that were greater than zero on average. The MIMOSA method modeled this structure through a Bayesian hierarchical mixture model framework. One component (or distribution) of the model represented the responders, and the other component modeled the non-responders. The parameters defining these distributions, as well as the probabilities that each ICS response was either a responder or non-responder, were estimated from the observed data. This sharing of information across SARS-CoV-2 responders and non-responders increased the sensitivity and specificity to make positivity calls [42]. Responses with probability of response > 0.999 were considered positive responders.

We considered nonlinear mixed effects models for T cell response, y_{ij}, as a function of t_{ij}, the j^{th} time since symptom onset for the i^{th} individual, with random effects for intercept and slope and $t_{ij} > 30$ days for all i, j:

$$\log_e y_{ij} = \beta_{0i} - \exp(\beta_{1i}) t_{ij} + \varepsilon_{ij}$$

where $\beta_{0i} = \beta_0 + b_i$ and $exp(\beta_{1i}) = exp(\beta_1 + c_i)$ with (b_i, c_i) iid $\sim N_2(0, \Sigma)$, with

$$\Sigma = \begin{bmatrix} \sigma_b^2 & 0 \\ 0 & \sigma_c^2 \end{bmatrix}$$

and σ_b^2 and σ_c^2 are the between-person variation in the intercept and slope of log T cell responses respectively, and ε_{ij} iid $\sim logNormal(0, \sigma^2)$. The random effects, b_i and c_i, are each assumed to be independent for different individuals and the within-individual errors ε_{ij} are assumed to be independent for different *i, j* and to be independent of the random effects. The function nlme from the R package nlme was used to fit the models.

Diagnostic plots of residuals were examined to assess validity of the model assumptions.

Age at enrollment, gender, and disease severity (WHO score > 4) were included as covariates in the mixed effects models to assess their association with each immune response.

Individual-level estimates at days 30 (T and B cell responses), day 42 (binding and neutralizing antibody responses) and day 180 (all responses) were obtained from the mixed effects models described above. Spearman rank correlations, Wald-based two-sided 95% confidence intervals and p values were reported.

Generalized estimating equations (GEE), with an independence working covariance matrix, were used to confirm the results of the covariate assessments for B and T cell responses from the mixed effects models. Two-tailed P values based on the robust standard error estimates for the covariate coefficients were consistent with the corresponding two-tailed P values for the covariate associations from the mixed effects models.

All tests were two-sided and P values < 0.05 were considered statistically significant unless otherwise noted. Details of specific statistical analyses can be found in the Results section and in the Figure legends.

Cell Reports Medicine, Volume *2*

Supplemental information

Longitudinal analysis shows durable and broad immune memory after SARS-CoV-2 infection with persisting antibody responses and memory B and T cells

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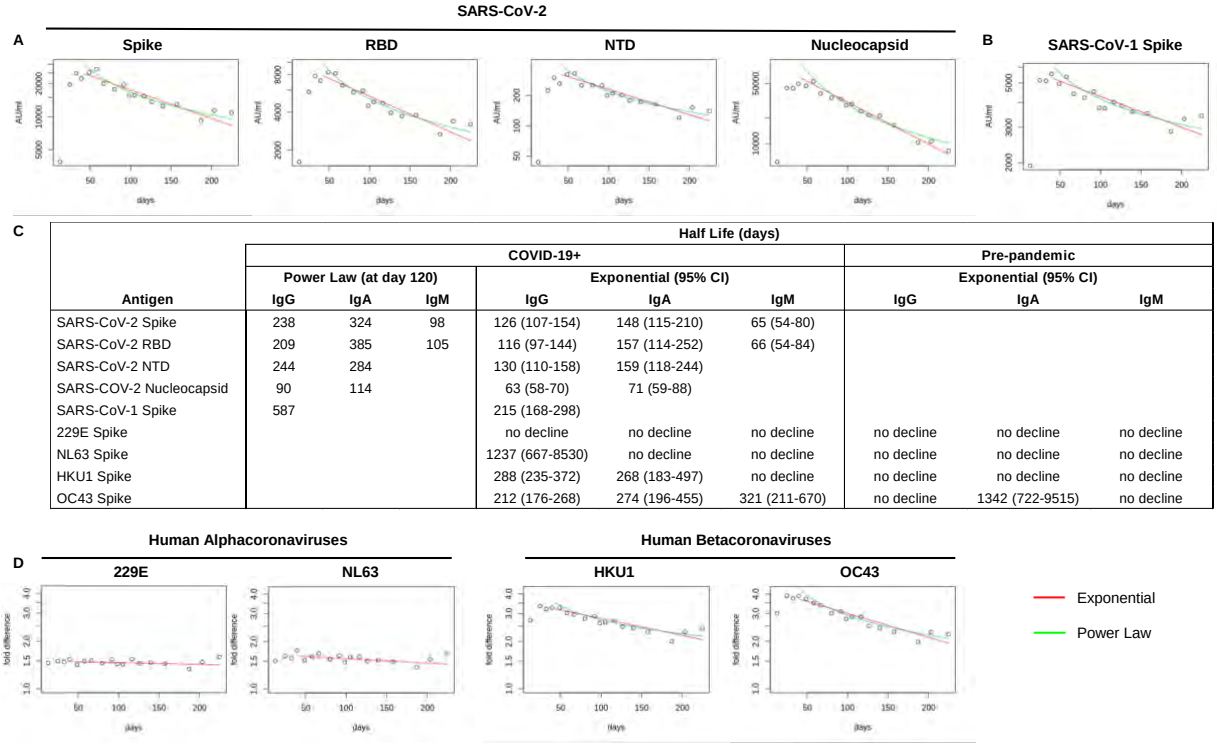
Table S1. Cohort Demographics and Baseline Characteristics (Related to STAR Methods Subject Details).

Characteristic	All (N=254)
Age, median (range)— years	48.5 (18-82)
Female sex at birth— no. (%)	141 (55.6)
Race or ethnic group— no. (%)	
White	226 (89.0)
Hispanic or Latino	21 (8.3)
Black or African American	15 (5.9)
Asian	11 (4.3)
Other[a]	7 (2.8)
Median time from symptom onset to enrollment (range)— days	53.5 (1-203)
Comorbid conditions— no. (%)	
Hypertension	46 (18.1)
Obesity	41 (16.1)
Chronic lung disease	23 (9.3)
HIV-1 and/or autoimmune disease	19 (7.7)
Type 2 diabetes mellitus	18 (7.3)
Heart disease	15 (6.0)
Cancer	10 (3.9)
Symptoms with initial illness— no. (%)	
Myalgia, fatigue	231 (90.9)
Headache	168 (66.1)
Fever	167 (65.7)
Cough	161 (63.4)
Loss of smell	146 (57.5)
Loss of taste	143 (56.3)
Shortness of breath	108 (42.5)
Diarrhea	102 (40.2)
Sputum production	43 (16.9)
None	9 (3.5)
Disease severity (WHO Score)—no. (%)	
Mild (1-2)	180[b] (70.9)
Moderate (3-4)	62 (24.4)
Severe (5-10)	12 (4.7)
Maximum number of visits—total	
1	9
2	103
3	62
4	51
5-7	29

[a]Individuals identifying as Other included: American Indian or Alaska Native; White (n=1); Asian, Black or African American (n=1); Asian; White (n=3); Native Hawaiian or other Pacific Islander; White (n=2); [b]6 participants had a positive Abbott SARS-CoV-2 Abbott SARS-CoV-2 IgG assay test but did not have a positive nasal SARS-CoV-2 PCR test.



	Half Life (days)								
	COVID-19+						Pre-pandemic		
	Power Law (at day 120)			Exponential (95% CI)			Exponential (95% CI)		
Antigen	IgG	IgA	IgM	IgG	IgA	IgM	IgG	IgA	IgM
SARS-CoV-2 Spike	238	324	98	126 (107-154)	148 (115-210)	65 (54-80)			
SARS-CoV-2 RBD	209	385	105	116 (97-144)	157 (114-252)	66 (54-84)			
SARS-CoV-2 NTD	244	284		130 (110-158)	159 (118-244)				
SARS-COV-2 Nucleocapsid	90	114		63 (58-70)	71 (59-88)				
SARS-CoV-1 Spike	587			215 (168-298)					
229E Spike				no decline	no decline	no decline	no decline	no decline	no decline
NL63 Spike				1237 (667-8530)	no decline	no decline	no decline	no decline	no decline
HKU1 Spike				288 (235-372)	268 (183-497)	no decline	no decline	no decline	no decline
OC43 Spike				212 (176-268)	274 (196-455)	321 (211-670)	no decline	1342 (722-9515)	no decline

Figure S1. Modeling of antibody titer decline. Decline of IgG antibody titers was analyzed by an exponential decay model (red) and a power law model (green) for antibodies reactive to SARS-CoV-2 antigens (A) and SARS-CoV-1 spike (B). The half-lives estimated by the exponential and power law models (C). The half-lives estimated by the power law were calculated at day 120 after symptom onset. The fold difference in IgG antibody titers to endemic coronaviruses between COVID-19 patients and pre-pandemic controls plotted over days since symptom onset (D). Related to Figure 1 and 2.

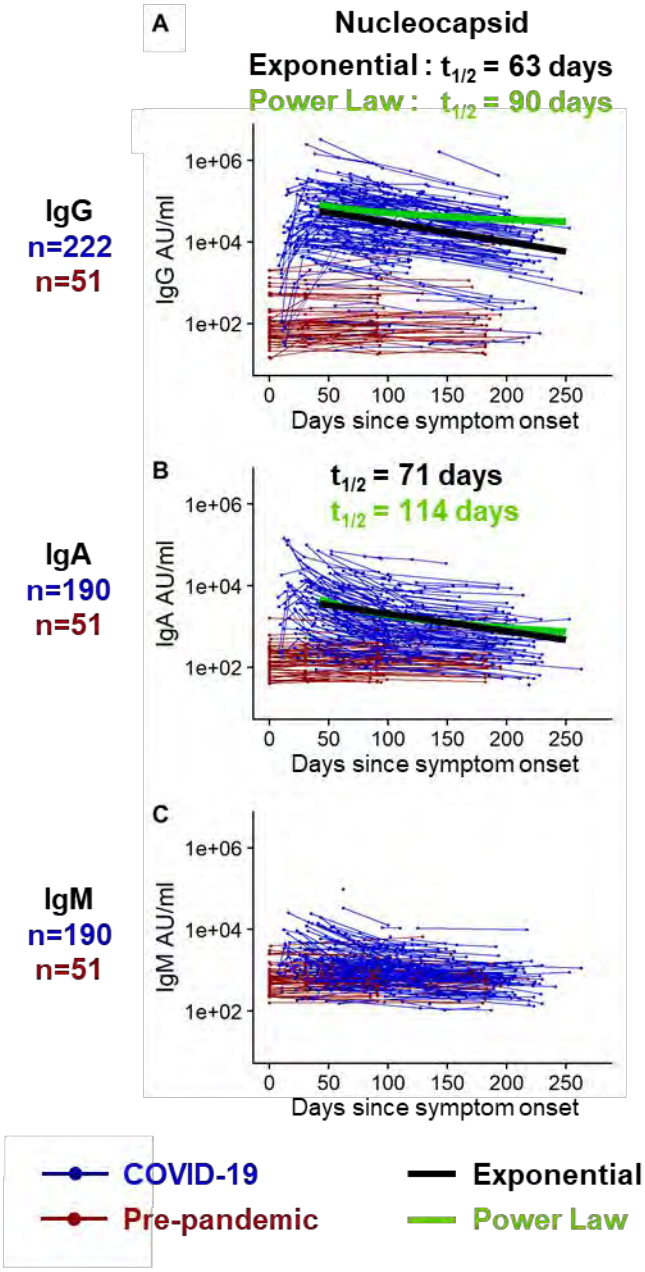


Figure S2. Longitudinal SARS-CoV-2 nucleocapsid binding antibody responses. IgG (A), IgA (B), and IgM (C) antibodies reactive to SARS-CoV-2 nucleocapsid were measured by an electrochemiluminescent multiplex immunoassay in triplicate and reported as arbitrary units per ml (AU/ml) as normalized by a standard curve. Longitudinal antibody titers of COVID-19 patients (in blue, n=222 COVID-19+ for IgG; n=190 COVID-19+ for IgA and for IgM) are plotted over days since symptom onset, whereas longitudinal pre-pandemic donor samples (in red, n=51 for IgG, IgA and IgM) were collected in the course of a non-SARS-CoV-2 vaccine study before 2019 and plotted over days since immunization. IgG decay curves and half-lives estimated by an exponential decay model are shown in black, whereas the decay curves and half-lives at day 120 post symptom onset estimated by a power law model are shown in green. Related to Figure 1.

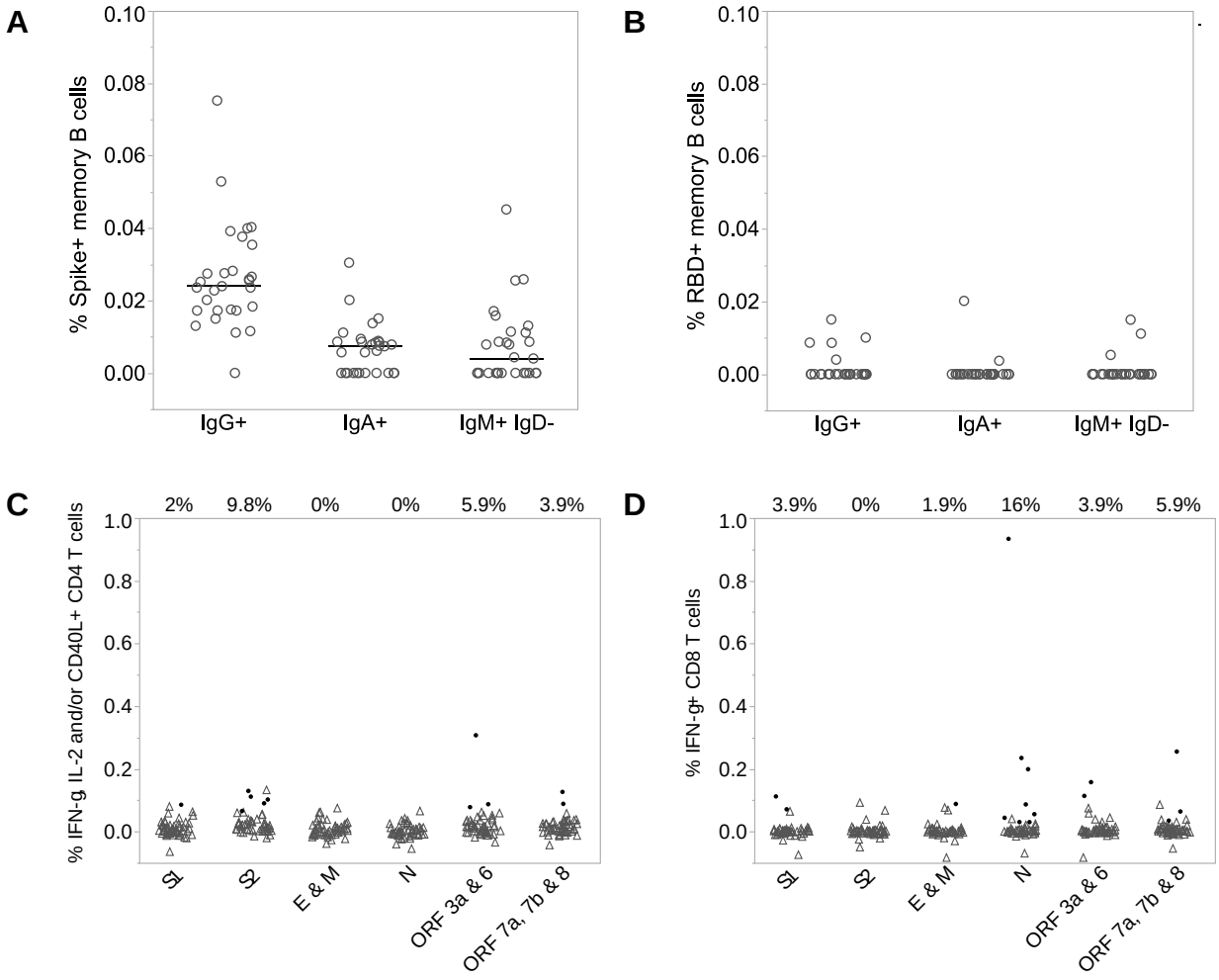


Figure S3. SARS-CoV-2 uninfected controls have few if any memory B and T cells recognizing SARS-CoV-2 antigens. Spike+ (A) and RBD+ (B) IgG+, IgA+ and IgM+ memory B cells in SARS-CoV-2 negative subjects are shown from PBMC collected before 2019 (n=29; tested in singlet). Line is at the median. Low frequencies of T cells recognizing SARS-COV-2 antigens are shown from donor samples not infected with SARS-CoV-2 (n=51). Background-subtracted CD4+ T cells expressing IFN-γ, IL-2 and/or CD40L (C), and IFN-γ+ CD8+ T cells (D) in response to stimulation with the SARS-CoV-2 antigens (on the x-axis) are shown. Positive T cell stimulations (as determined by MIMOSA) are indicated by a solid black circle, whereas samples that are negative are indicated by gray open triangles and the percent of positive responders are shown above the T cell graphs. Related to Figure 4, 5 and 6.

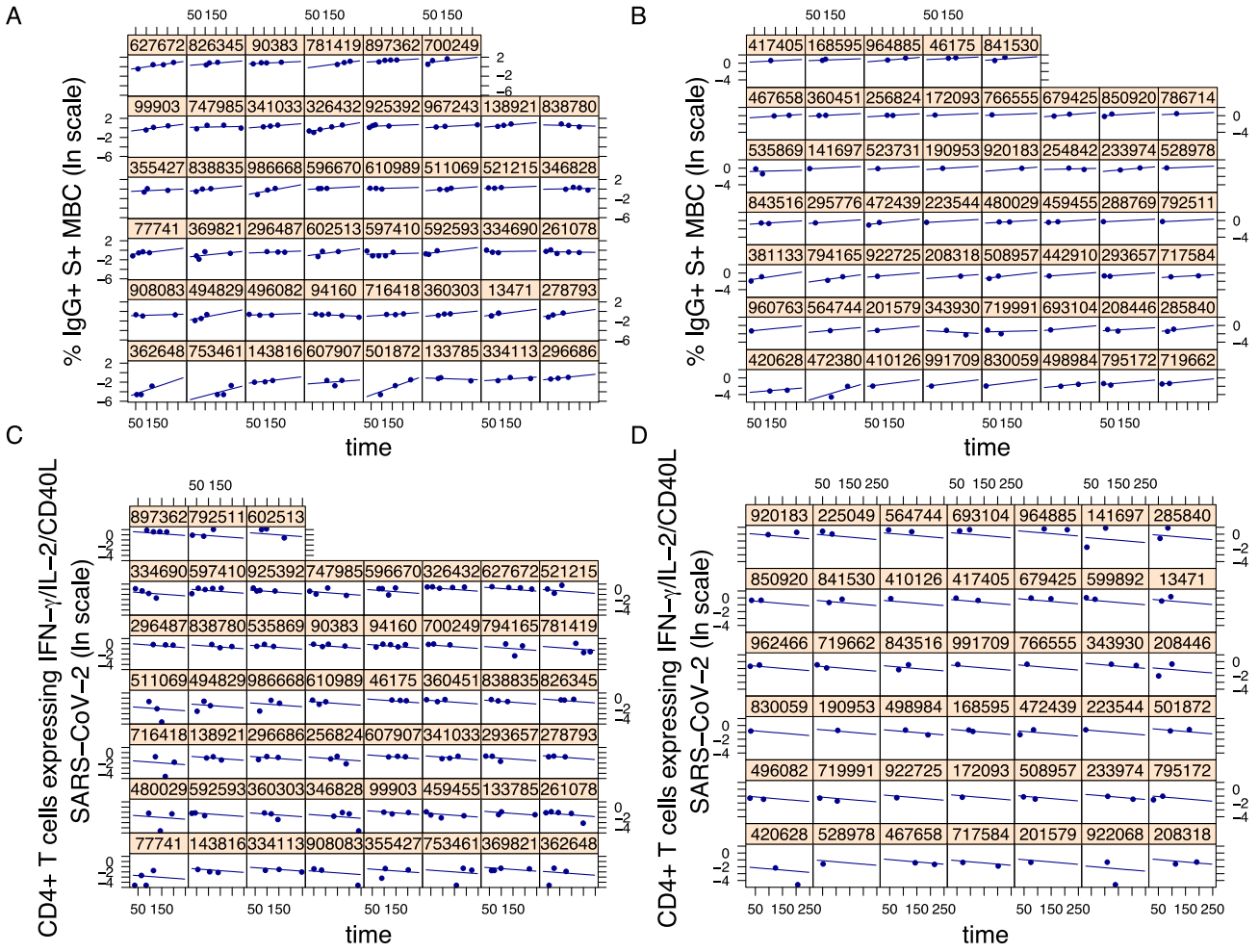


Figure S4. Representative individual-level estimates of SARS-CoV-2 B and T cell responses from 30 days post-symptom onset. Post-day 30 S+ IgG+ B cell responses ($\log_e$ scale) for individuals with data at 3 or more time points (A) and 1-2 time points (B) with fitted curves from a linear mixed effects model with random effects for the intercept and slope. Post-day 30 CD4+ T cell responses to SARS CoV-2 ($\log_e$ scale) for individuals with data at 3 or more time points (C) and 1-2 timepoints (D), with fitted curves from a nonlinear mixed effects model with random effects for the intercept and slope. The CD4+ T cell analyses only included individuals with a positive response to a least one SARS-CoV-2 antigen at one or more time points, where positive responses were determined by MIMOSA. Related to Figures 4 and 5.

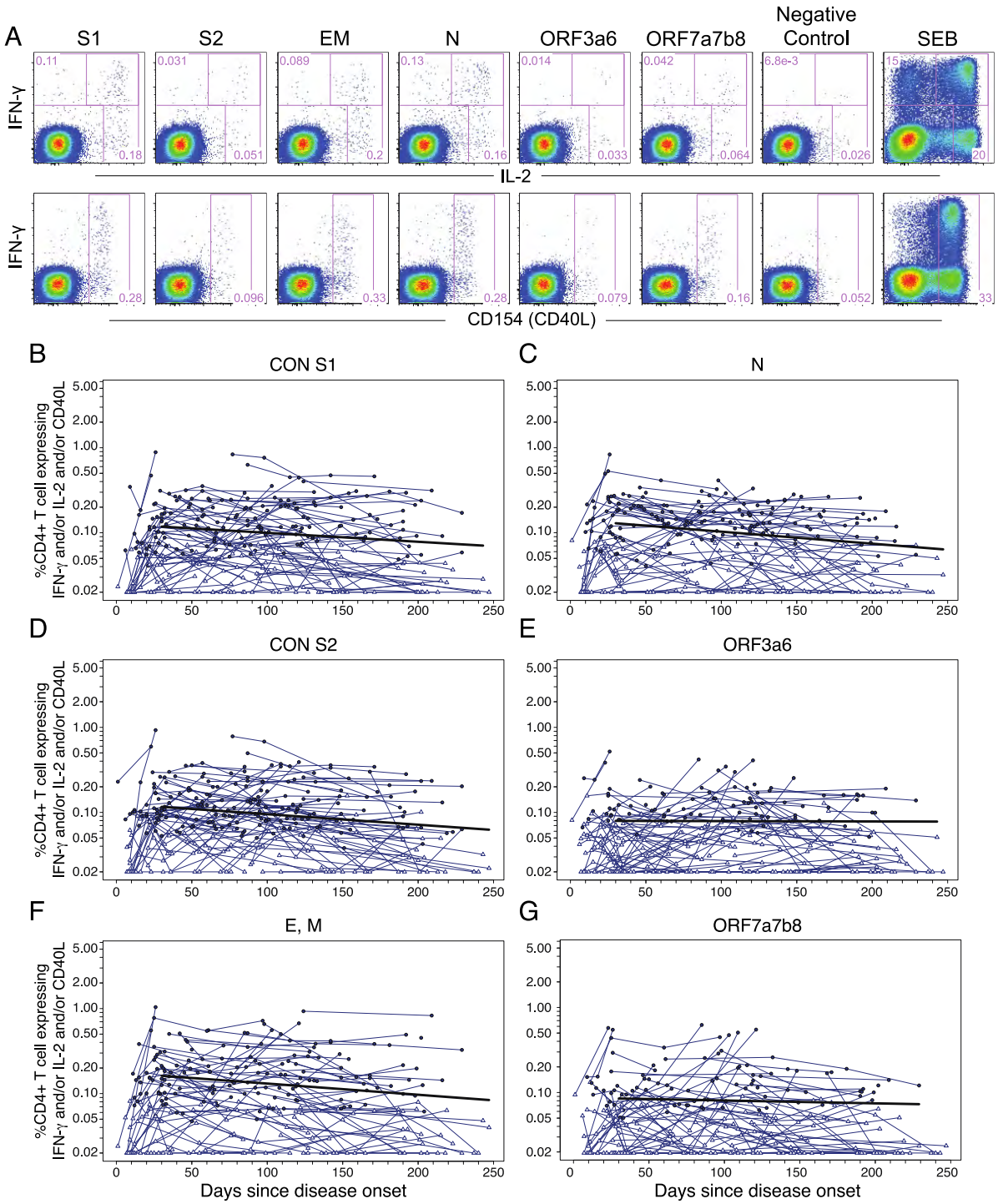


Figure S5. CD4+ T cell responses among SARS-CoV-2 convalescent subjects to individual SARS-CoV-2 peptide pools. (A) Representative SARS-CoV-2 specific CD4+ T cell responses to multiple SARS-CoV-2 antigens by intracellular cytokine staining (ICS) assay in PBMCs from a SARS-CoV-2 patient. Background-subtracted frequencies of IFN-γ+, IL-2+ and/or CD40L+ CD4+ T cells responding to: (B) S1, (C) S2, (D) envelope and membrane (EM), (E) N, (F) ORF3a and 6, (G) ORF7a, 7, and 8 (n=114; tested in single replicates). Positive responses as determined by MIMOSA are indicated by a solid circle and negative responses are indicated by open triangles. The bold black line represents the median fitted curve from a nonlinear mixed effects model of post-day 30 responses with random effects for the intercept and slope. The mixed effects models only include individuals with a positive response to the antigen(s) under consideration at one or more time points. Related to Figure 5.

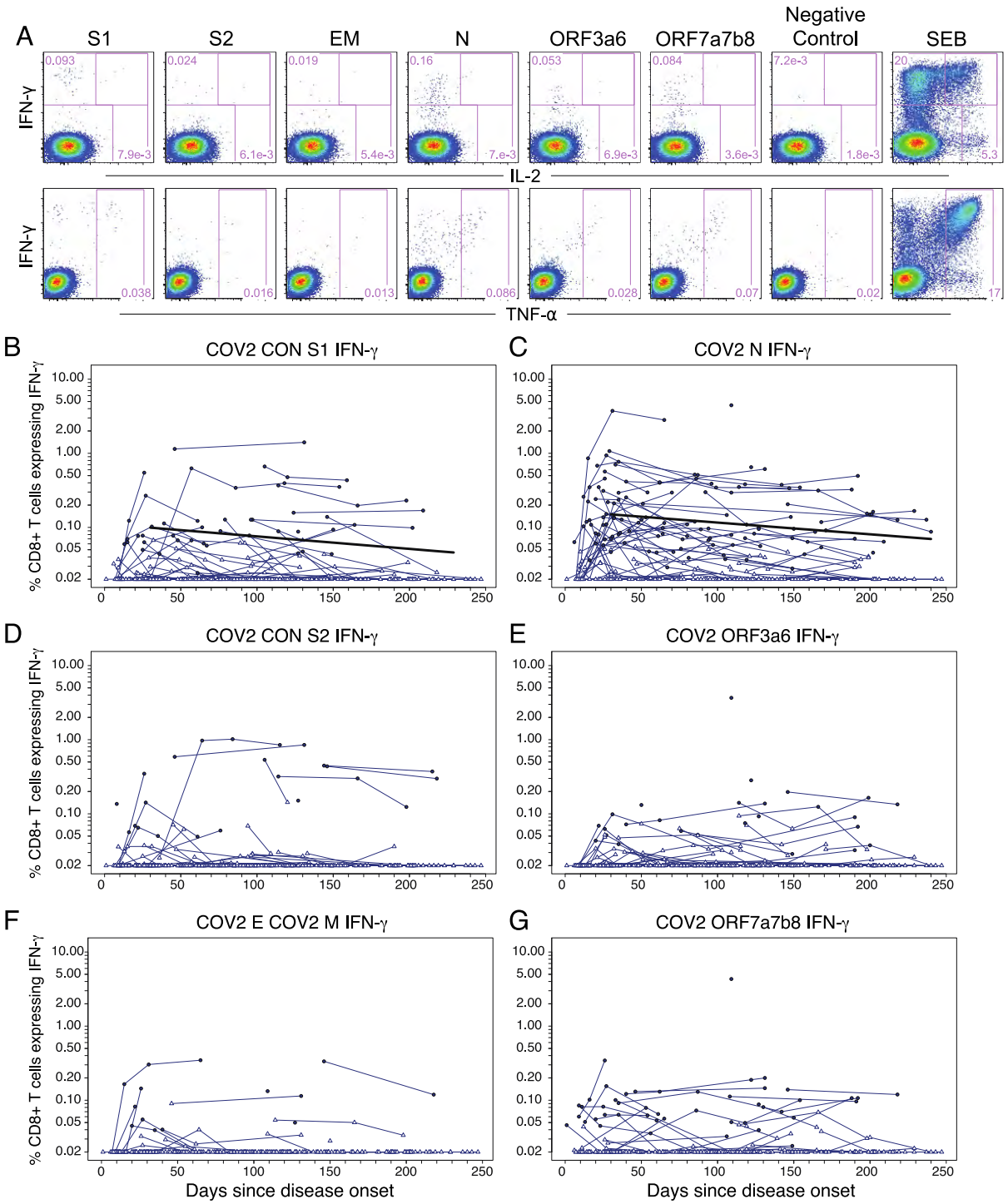


Figure S6. CD8 T+ cell responses among COVID-19 patients to individual SARS-CoV-2 peptide pools. (A) Representative SARS-CoV-2-specific CD8+ T cell responses to multiple SARS-CoV-2 antigens by intracellular cytokine staining (ICS) assay in PBMCs from a SARS-CoV-2 patient. Background-subtracted frequencies of IFN-γ+ CD8+ T cells responding to: (B) S1, (C) S2, (D) envelope and membrane (EM), (E) N, (F) ORF3a and 6, (G) ORF7a, 7, and 8 (n=114; tested in single replicates). Positive responses as determined by MIMOSA are indicated by a solid circle, and negative responses are indicated by open triangles. The bold black line represents the median fitted curve from a nonlinear mixed effects model of post-day 30 responses with random effects for the intercept and slope. The mixed effects models only included individuals with a positive response to the antigen(s) under consideration at one or more time points. Related to Figure 6.

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Original Research

Clinical outcomes after early ambulatory multidrug therapy for high-risk SARS-CoV-2 (COVID-19) infection

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There is an emergency need for early ambulatory treatment of Coronavirus Disease 2019 (COVID-19) in acutely ill patients in an attempt to reduce disease progression and the risks of hospitalization and death. Such management should be applied in high-risk patients age $>$ 50 years or with one or more medical problems including cardiovascular disease. We evaluated a total of 922 outpatients from March to September 2020. All patients underwent contemporary real-time polymerase chain reaction (PCR) assay tests from anterior nasal swab samples. Patients age 50.5 $\pm$ 13.7 years (range 12 to 89), 61.6% women, at moderate or high risk for COVID-19 received empiric management via telemedicine. At least two agents with antiviral activity against SARS-CoV-2 (zinc, hydroxychloroquine, ivermectin) and one antibiotic (azithromycin, doxycycline, ceftriaxone) were used along with inhaled budesonide and/or intramuscular dexamethasone consistent with the emergent science on early COVID-19 treatment. For patients with high severity of symptoms, urgent in-clinic administration of albuterol nebulizer, inhaled budesonide, and intravenous volume expansion with supplemental parenteral thiamine 500 mg, magnesium sulfate 4 grams, folic acid 1 gram, vitamin B12 1 mg. A total of 320/922 (34.7%) were treated resulting in 6/320 (1.9%) and 1/320 (0.3%) patients that were hospitalized and died, respectively. We conclude that early ambulatory (not hospitalized, treated at home), multidrug therapy is safe, feasible, and associated with low rates of hospitalization and death. Early treatment should be considered for high-risk patients as an emergency measure while we await randomized trials and guidelines for ambulatory management.

Keywords

SARS-CoV-2; COVID-19; multidrug; hospitalization; mortality; ambulatory; antiviral; zinc; hydroxychloroquine; ivermectin; doxycycline; azithromycin; vitamin; corticosteroid

1. Background

The epidemic viral outbreak of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) infection Coronavirus Disease 2019 (COVID-19) is advancing across the United States unabated despite public policy measures focussed on contagion control (McCullough et al., 2020). The United States has a current 877 deaths per million inhabitants despite having technically advanced hospitals and to date sufficient capacity to handle the surges of patients requiring hospitalization (Worldometer, 2020). Conversely India, a country with broad implementation of early COVID-19 treatment has 102 deaths per million (Worldometer, 2020). The regulatory agencies as well as the National Institutes of Health have had their principal areas of focus being late stage hospitalized patients and vaccine development (COVID-19 Treatment Guidelines, 2020). This has left a void for the role of early ambulatory treatment of COVID-19 at home. Such management has the goals of lessening the intensity and severity of symptoms and preventing hospitalization and death. There are currently no approved drugs or drug combinations in the U.S. indicated for the ambulatory treatment of COVID-19 or its complications. In the absence of conclusive randomized trials of single drugs and combination regimens, clinicians faced with large numbers of ill patients have responded with innovative empiric approaches that attempt to reduce the progression of SARS-CoV-2 infection, improve symptoms, avoid complications, and reduce the risk of complications and death. The mechanisms by which a multidrug approach would globally improve outcomes could be to address viral replication, cytokine storm, and thrombosis. This report discloses real world data and the clinical outcomes of early ambulatory treatment of acute COVID-19 in patients at high risk for hospitalization and death.

2. Methods and results

Beginning in March 2020, a team of primary care providers consisting of a lead physician (BCP) and four advanced practice practitioners (CR, VP, ES, CH) responded to urgent visits by patients with suspected SARS-Co-V infection and symptomatic COVID-19. All patients underwent standard informed consent for

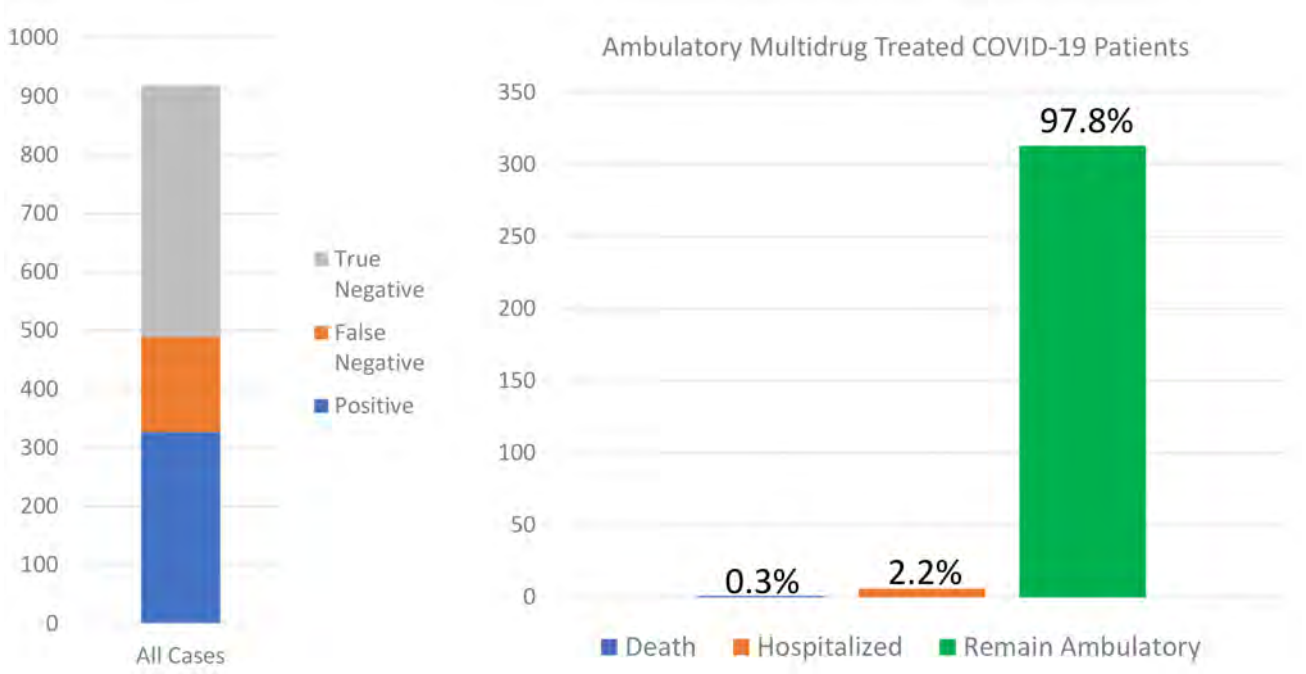

Fig. 1. The SARS-CoV-2 nasal PCR test results are shown on the left and among the 320 cases that were confirmed positive and were high risk, the outcomes of hospitalization and death are shown on the right.

Table 1. Symptom severity score for initial assessment of patients infected with suspected infection of SARS-CoV2 (COVID-19).

Symptom	Points
Fever	1
Fever at night	1
Fatigue	1
Body aches	1
Cough	1
Difficulty breathing	1
Additional symptoms	1
Probability of COVID-19: 0-1 points = low, 3-4 points = moderate, 5+ points = high	

care and were under the direct management of licensed medical personnel including a senior attending physician (BCP). Contemporary real-time polymerase chain reaction (PCR) assay tests from anterior nasal swab samples were obtained. It was understood at that time period that COVID-19 test results could be falsely negative, particularly in the setting where a patient had the characteristic symptoms of the syndrome (Woloshin et al., 2020). They additionally had an assessment according to the severity of symptoms and scored as depicted in Table 1. The treatment regimens are given in Table 2. All patients received empiric treatment on the first day of presentation in most cases before COVID-19 test results with standard office practice and contagion control measures (Fiorillo et al., 2020). According to clinical judgment and planned use of hydroxychloroquine (HCQ) a 12-lead electrocardiogram was obtained to evaluate the QTc interval. For patients with high severity of symptoms, urgent in-clinic administration of albuterol nebulizer, inhaled budesonide, and intravenous volume expansion with supplemental parenteral thiamine 500 mg, magnesium sulfate 4 grams, folic acid 1 gram, vitamin B12 1 mg (Flannery et al., 2017). Additionally, for the severely ill population dexamethasone 8 mg and ceftriaxone 1 gram was administered intramuscularly (Table 2). All patients had in-person or telemedicine followup at 48 hours and as needed after that point which was part of the general consent for treatment. Univariate statistics were reported with means $\pm$ standard deviation or counts with proportions as appropriate.

A total of 922 patients were evaluated between the ages of 12 and 89 years. The mean age was 50.5 $\pm$ 13.7 years and 61.6% were women. The frequency of comorbidities was as follows: obesity 60.5%, diabetes mellitus 10%, cardiovascular disease 33.7%, pulmonary illness 17.8%. The rate of positive SARS-CoV-2 positive tests was 327/918 (35.6%). Among the 591 test negative patients, 162 (27.4%) were considered false negative tests since they went on to develop persistent or worsening symptoms of COVID-19. A total of 320/922 (34.2%) were treated based on age $>$ 50 and/or the presence of comorbidities (obesity, diabetes mellitus, cardiopulmonary disease, chronic kidney disease, etc). All patients were followed for a minimum of 90 days. Clinical outcomes included 6/320 (1.9%) and 1/320 (0.3%) that were hospitalized or died, respectively (Fig. 1).

3. Discussion

The observations in this report suggest that primary care physicians can take an organized, empiric approach to acutely ill patients with COVID-19 with very low rates of subsequent hospitalization and death. The execution of this program was heavily dependent on telemedicine technology (Cervino and Oteri, 2020). Our observations suggest a majority of hospitalizations could be

Table 2. Combination medications for a minimum of five days and acutely administered supplements used for the initial ambulatory patient with suspected and or confirmed COVID-19 (moderate or greater probability).

Agent	Rationale
Zinc	Inhibits SARS-CoV-2 RNA synthesis
Hydroxychloroquine 200 mg po bid	Inhibits endosomal transfer of virions, anti-inflammatory
Ivermectin (200 mcg/kg) usual dose 12 mg po qd × 3 days	Attenuates importin α/β-mediated nuclear transport of SARS-CoV-2 into nucleus
Azithromycin 250 mg po bid	Covers respiratory bacterial pathogens in secondary infection
Doxycycline 100 mg po bid	Covers respiratory bacterial pathogens in secondary infection
Inhaled budesonide, Dexamethasone 8 mg IM	Treats cytokine storm
Folate, thiamine, vitamin 12	Reduce tissue oxidative stress
Intravenous fluid	Intravascular volume expansion

avoided and the spread of SARS-CoV-2 can be reduced with a first treat-at-home approach featuring telemedicine during follow-up (Gambardella et al., 2020; Tolone et al., 2020). We leveraged of agents that were commercially available and had a reasonable chance of therapeutic gain with acceptable safety. Because multiple agents are used empirically and in combination given the context of an emergency pandemic, it is impossible to retrospectively stratify for each component and analyze individual effects. We addressed viral replication, cytokine storm, and tissue damage due to oxidative stress utilizing vitamins, micronutrient supplements, and prescription medications (Zhang et al., 2020). Additionally, we encouraged the use of renin-angiotensin system inhibitors based on their theoretical effect over the long term for upregulation of the angiotensin converting enzyme 2 receptor which, despite being the entry receptor for the SARS-CoV-2 also protects lungs in preclinical models of adult respiratory distress syndrome (Lo et al., 2020; Palazzuoli et al., 2020). Our approach was later supported by concurrent analyses and subsequent published reports (Derwand et al., 2020; Lo et al., 2020; Palazzuoli et al., 2020). The observed rates of these outcomes are considerably lower than reported in other studies in our region. A recent report from Methodist hospital in Houston reported that patients with progressive symptoms when hospitalized suffered a 5.8% mortality rate was despite the use of HCQ, remdesivir, convalescent plasma, and anticoagulants (Vahidy et al., 2020). Undoubtedly a portion of the mortality benefit of outpatient therapy is reducing the need for supplemental oxygen and mechanical ventilation. A recent series from Italy has demonstrated that there is a graded increase in death rates with 7.4% for whom no oxygen was required, 12.9% for oxygen-requiring, and 23.0% for mechanically ventilated patients (Palazzuoli et al., 2020). Our data suggest the advancement of early home use of off-target antiviral agents (zinc, HCQ, ivermectin, azithromycin, doxycycline), antibiotics, corticosteroids, and in the future empiric anticoagulants could markedly reduce the risk for hospitalization and potentially reduce overall death rates before and during hospitalization (McCullough et al., 2020).

Our report has all the limitations common to the reporting of clinical practice outcomes. During this time there was an evolving set of SARS-CoV-2 assays, and hence when assessed in context to the clinical syndrome, we experienced both false positive and negative testing as reported. Follow-up was performed by usual practice call logs and electronic medical systems and is temporally truncated to the time of this report.

4. Conclusions

In conclusion, empiric multidrug treatment for ambulatory COVID-19 according to age, comorbidities, and initial severity of symptoms is feasible with close follow-up. Our data suggest that such a strategy is associated very low rates of hospitalization in high-risk patients who receive early outpatient treatment. This may be due to symptom relief attributed to medications, supportive parenteral volume expansion, micronutrient supplementation, and compassionate care delivered by in person visits and telemedicine. The rates of death in our study indicate that early multidrug therapy is associated with $>$ 90% reduction in mortality among the high risk compared to community rates of death associated with therapeutic nihilism in ambulatory patients who are subsequently hospitalized. The National Institutes of Health currently advise denial of early treatment and encourage late-stage hospitalization as the first window of treatment open to acutely ill patients with COVID-19 (COVID-19 Treatment Guidelines, 2020). Our contrary view, supported by our results, is that early ambulatory therapy should be offered as an emergency measure in acutely ill, high-risk COVID-19 as a strategy to reduce hospitalization and death. We anticipate results of clinical trials will refine our multipronged therapeutic response to patients with this potentially fatal infection, however in our view, there is an impressive and urgent call for action at the earliest point in the infection for the best chances of survival from hospitalization and death.

Authors' contributions

BCP, CR, VP, ES, CH, contributed patient data, PAM drafted the first version, and all authors contributed edits to the final version.

Ethics approval and consent to participate

All patients provided informed consent for treatment according to good clinical practice.

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There are no acknowledgments to disclose.

Conflict of interest

Nothing to disclose. Authors had access to the data and wrote the manuscript.

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Avoiding pitfalls in the pursuit of a COVID-19 vaccine

As they race to devise a vaccine, researchers are trying to ensure that their candidates don't spur a counterproductive, even dangerous, immune system reaction known as immune enhancement.

Lynne Peeples, *Science Writer*

The teams of researchers scrambling to develop a coronavirus disease 2019 (COVID-19) vaccine clearly face some big challenges, both scientific and logistical. One of the most pressing: understanding how the immune system interacts not only with the pathogen but with the vaccine itself—crucial insights when attempting to develop a safe and effective vaccine.

Researchers need to understand in particular whether the vaccine causes the same types of immune system malfunctions that have been observed in past vaccine development. Since the 1960s, tests of vaccine candidates for diseases such as dengue, respiratory syncytial virus (RSV), and severe acute respiratory syndrome (SARS) have shown a paradoxical phenomenon: Some animals or people who received the vaccine and were later exposed to the virus developed more severe disease than those who had not been vaccinated (1). The vaccine-primed immune system, in certain cases, seemed to launch a shoddy response to the natural infection. "That is something we want to avoid," says Kanta Subbarao, director of the World Health Organization Collaborating Centre for Reference and Research on Influenza in Melbourne, Australia.

SARS-CoV-2—the virus that causes COVID-19 and the focus of numerous vaccine development efforts—has three surface proteins attached to a lipid bilayer, as seen in this illustration based on X-ray diffraction data. Image credit: Science Source/Juan Gaertner.

This immune backfiring, or so-called immune enhancement, may manifest in different ways such as antibody-dependent enhancement (ADE), a process in which a virus leverages antibodies to aid infection; or cell-based enhancement, a category that includes allergic inflammation caused by Th2 immunopathology. In some cases, the enhancement processes might overlap. Scientific debate is underway as to which, if any, of these phenomena—for which exact mechanisms remain unclear—could be at play with the novel coronavirus and just how they might affect the success of vaccine candidates.

A vaccine is designed to boost our natural immune response to an invading virus by priming it to recognize antigens, unique molecules found on the surface of pathogens. Ideally, the immune system responds to the presence of these antigens by producing special immune cells that directly attack the pathogen, or by producing proteins called antibodies. Antibodies attach to an antigen and attract immune cells that engulf and destroy the pathogen. A dysregulated immune response may involve antibodies or immune cells—or both.

Some researchers argue that although ADE has received the most attention to date, it is less likely than the other immune enhancement pathways to cause a dysregulated response to COVID-19, given what is known about the epidemiology of the virus and its behavior in the human body. "There is the potential for ADE, but the bigger problem is probably Th2 immunopathology," says Ralph Baric, an epidemiologist and expert in coronaviruses—named for the crown-shaped spike they use to enter human cells—at the University of North Carolina at Chapel Hill. In previous studies of SARS, aged mice were found to have particularly high risks of life-threatening Th2 immunopathology (2). Baric expresses his concern about what that might mean for use of a COVID-19 vaccine in

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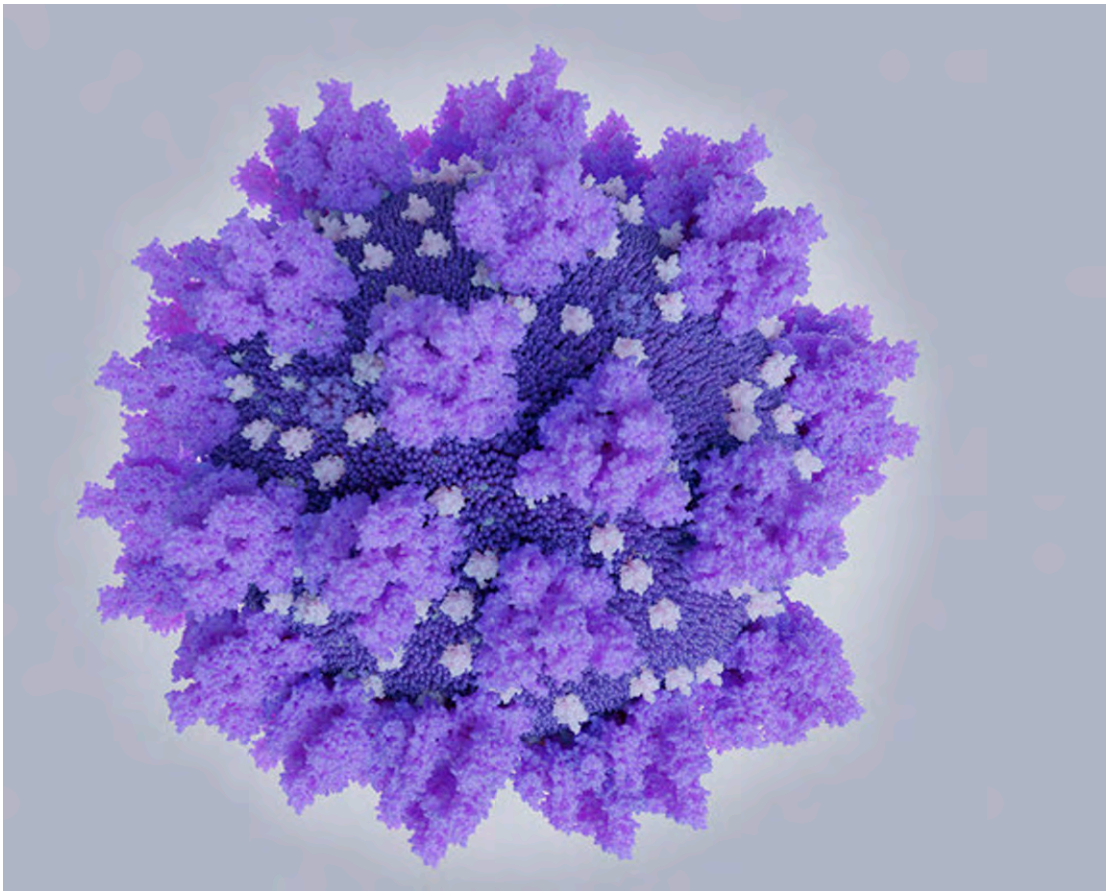

Researchers are debating which, if any, of the phenomena related to immune enhancement could be at play in the case of the novel coronavirus—and just how these phenomena might affect the success of vaccine candidates. Image credit: Shutterstock/PhotobyTawat.

elderly people. "Of course, the elderly are our most vulnerable population," he adds.

Experts generally agree that animal experiments and human clinical trials of candidate vaccines for COVID-19, which is caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), should include a careful assessment of possible immune complications before releasing the vaccine to the public. If any of the mechanisms under investigation are indeed involved, they say, the resulting risks are real. "You really have to test a vaccine carefully," says Marc Lipsitch, an epidemiologist at the Harvard Chan School of Public Health in Boston, MA, "and not just roll it out because people are clamoring for it with an epidemic underway."

Picking the Right Problem

Upwards of 80% of patients who contract COVID-19 develop only mild flu-like symptoms. "The immune system fights off the virus and people might hardly notice," says Darrell Ricke, a researcher with the MIT Lincoln Laboratory's Bioengineering Systems and Technologies Group in Lexington, MA, who posted a preprint in March on the possible COVID-19 vaccine risks (3). "But there seems to be a tipping point: Some individuals appear equally healthy yet can progress to a more severe disease."

Ricke points to ADE as a potential explanation for this variability. The phenomenon has been reported in some tissue culture and animal studies of HIV, influenza, and SARS. But it is best known for its influence on the immune response to the dengue virus. If a person is infected with one of dengue's four serotypes, their immune system should confer lifelong protection against that serotype. But as researchers have discovered, if that person is later infected by a different dengue serotype, then they can develop a severe and potentially deadly illness. In fact, according to one study in the 1980s, more severe responses were found to be 15 to 80 times more likely in secondary dengue infections than in primary infections (4). Instead of the antibodies neutralizing encountered dengue viral proteins, they enhance uptake of the virus. The back end of the antibody binds to macrophages, a type of white blood cell, and helps the virus enter those cells and accelerate viral replication.

ADE has posed a similar challenge in the creation of vaccines for infections including dengue and a cat coronavirus, feline infectious peritonitis virus (FIPV). In one study, cats vaccinated against FIPV got sicker than cats left unvaccinated (5). Again, the virus-specific antibody increased the virus uptake by macrophages.

Yet some experts doubt that ADE is relevant for COVID-19. "We have no evidence that ADE is actually

occurring in human patients," says Angela Rasmussen, a virologist at Columbia University Mailman School of Public Health in New York, citing such findings.

In principle, anecdotal reports of COVID-19 reinfections in China (6) could lend credence to relevance of ADE—that is, the production of antibodies to the virus (resulting from immunization or an initial natural infection) ends up enhancing entry of the virus into cells. But Rasmussen and other experts underscore the lack of real evidence for COVID-19 reinfections. Any repeat cases so far reported, they say, could be explained by false negative tests between the positive tests. "It's not clear that patients were ever not infected," says Rasmussen.

And there is some preliminary experimental evidence casting doubt on ADE. Two papers published in March in *Cell* show that antibodies against the original SARS infection, which emerged in China in 2002, could also block entry of SARS-CoV-2 into human cells. Another preprint study showed that rhesus macaques infected with SARS-CoV-2 and allowed to recover were not infected after a second exposure to the virus. Unless future data correlate severe COVID-19 cases with original SARS infections—or other diagnostic, pathology, or clinical findings indicate ADE—then there is "not much to go on that suggests ADE is a factor," Rasmussen says.

Barney Graham, deputy director of the Vaccine Research Center at the National Institute of Allergy and Infectious Diseases, in Bethesda, MD, which is collaborating with the Cambridge, MA-based biotech Moderna on a COVID-19 vaccine candidate, also questioned the role of ADE. Dengue is a flavivirus, a family of viruses that are known to infect macrophages. FIPV also infects macrophages. ADE is unlikely to occur in the current coronavirus, Graham argues, because it does not target or grow in macrophages. Rather, SARS-CoV-2 primarily infects the respiratory epithelial cells, which present different receptors.

"Ecological disruption really increases the odds that we might encounter a pathogen that we've never seen before but grows in us just fine."

—Angela Rasmussen

Rogue Responses

Graham emphasizes alternative ways in which a vaccine could potentially induce more serious COVID-19 infections: Th2 immunopathology, in which a faulty T cell response triggers allergic inflammation, and poorly functional antibodies that form immune complexes, activating the complement system and potentially damaging the airways.

Both processes were at play as an unfortunate situation unfolded in the 1960s, according to Graham. Researchers at the time were pursuing a vaccine against RSV, the leading cause of severe respiratory illness in infants. In trials of one vaccine candidate, several children who received the vaccine developed a serious illness when infected with the natural virus (7). Two toddlers died. In this case, researchers noticed severe damage and the unexpected presence of lots of neutrophils and eosinophils, both immune cells, in the children's lung tissue. A similar inflammatory response was seen in animal models of RSV, in which cytokines, a type of immune cell, had invaded and damaged tissue.

"That really killed RSV vaccines for a generation," says Peter Hotez, a vaccine researcher and dean of the National School of Tropical Medicine at Baylor College of Medicine in Houston, TX. After more than 50 years of further study, a candidate RSV vaccine is finally back in clinical trials.

When SARS, also a coronavirus, appeared in China and spread globally nearly two decades ago, Hotez was among researchers who began investigating a potential vaccine. In early tests of his candidate, he witnessed how immune cells of vaccinated animals attacked lung tissue, in much the same way that the RSV vaccine had resulted in immune cells attacking kids' lungs. "I thought, 'Oh crap,'" he recalls, noting his initial fear that a safe vaccine may again not be possible.

But his team revised their approach. Instead of producing the whole spike protein of the virus, they built just a tiny piece of it—the piece that attaches to human cells, called the receptor-binding domain. Subsequent animal tests showed that this strategy did provide the desired protection without the unwanted immune enhancement. With funding from the NIH, Hotez's team continued on to manufacture the vaccine and were ready for clinical trials.

False Start

But then they hit a roadblock. The money dried up. By that time, SARS was no longer spreading, and interest in a vaccine had waned. In the face of the current pandemic coronavirus, Subbarao suggests, that "risk–benefit calculation might be very different."

Indeed, when COVID-19 appeared in China, Hotez took special notice, in part because it belonged to the coronavirus family. "I thought we may be sitting on a valuable vaccine. I think it could partially cross-protect against both viruses," he says. "And the exciting part is I think we have already partially solved the immune enhancement problem."

Hotez is currently seeking funding for clinical trials of the original vaccine, while also working to produce a new vaccine for COVID-19. Although the basic machineries of the two coronaviruses are nearly identical, the team will need to make adjustments for the slight differences in receptor-binding domains between SARS-CoV-1 and SARS-CoV-2. Ricke notes that the outer surface of the spike protein has been remodeled by mutations that, over time, have made it a better binder and more infective as an airborne pathogen.

Antibodies produced to bind to the original SARS antigens may not bind as consistently to the new SARS antigens. And that lack of potency could raise the risk of immune enhancement, suggests Graham.

Otherwise, he says, the receptor-binding domain approach could be effective.

Hotez and others believe that the vaccine lag for COVID-19 might have been avoided if candidate vaccines for SARS or Middle East Respiratory Syndrome (MERS) had received clinical trial funding years ago. "If we had already had a licensed human coronavirus vaccine, we would be a lot less worried about these safety concerns. Because we don't have one, we're in new territory," says Subbarao. "When we work on pandemic influenza vaccines we have years of experience with influenza vaccines [and] we can build from that."

Not So Fast

Still, several teams are working in parallel with a diverse set of strategies to develop a potent—and hopefully harmless—vaccine.

Graham's team is attempting to mitigate the possibility of immune enhancement and maximize the speed of vaccine development by injecting mRNA in order to make a highly precise type of protein. "We know at atomic-level detail that this protein is shaped the right way to elicit the right antibodies to have functional activity against this virus," says Graham. "These things create the kind of T cell response that will prevent allergic inflammation." Another perk of gene-based delivery: It can be manufactured rapidly.

Moderna's mRNA vaccine candidate has progressed at unprecedented speed, thanks in large part to China's January release of the genetic sequence of the virus. A phase 1 clinical trial began on March 16 in Seattle, WA. "We need to get some answers by next winter so we can at least be more prepared for the winter of 2021–2022," adds Graham.

But immune enhancement concerns linger. Stanley Perlman, a professor of microbiology and immunology at the University of Iowa in Iowa City, agrees that a good T cell response should sidestep enhancement concerns. He is also part of a special committee convened by the World Health Organization (WHO) to address immune enhancement, which they refer to as vaccine enhancement. The committee now aims to define what exactly this enhancement means, what the relevant issues are for a COVID-19 vaccine, and what to do with that information, notes Perlman. A subgroup of the committee is expected to produce a summary report within a few months.

Given how many vaccine candidates are now in the running, Rasmussen says she is confident that at least one of them will work. "But, by the time they have gone through trials to determine safety and efficacy, will there be the same kind of public will to push this out on the market?" she says. "Will we continue to have government and private industry investments?" The WHO and other health leaders emphasize that it will likely be a year and a half before a vaccine is vetted through trials in animals and humans and ready for dissemination.

Vaccine experts have underscored the need to avoid mistakes from the past, such as the halting of SARS vaccine development. More coronaviruses are likely waiting in wild bats, primates, and rodents, ready to make the jump to humans. "Ecological disruption really increases the odds that we might encounter a pathogen that we've never seen before but grows in us just fine," says Rasmussen.

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Review of the Emerging Evidence Demonstrating the Efficacy of Ivermectin in the Prophylaxis and Treatment of COVID-19

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Background: After COVID-19 emerged on U.S shores, providers began reviewing the emerging basic science, translational, and clinical data to identify potentially effective treatment options. In addition, a multitude of both novel and repurposed therapeutic agents were used empirically and studied within clinical trials.

Areas of Uncertainty: The majority of trialed agents have failed to provide reproducible, definitive proof of efficacy in reducing the mortality of COVID-19 with the exception of corticosteroids in moderate to severe disease. Recently, evidence has emerged that the oral antiparasitic agent ivermectin exhibits numerous antiviral and anti-inflammatory mechanisms with trial results reporting significant outcome benefits. Given some have not passed peer review, several expert groups including Unitaid/World Health Organization have undertaken a systematic global effort to contact all active trial investigators to rapidly gather the data needed to grade and perform meta-analyses.

Data Sources: Data were sourced from published peer-reviewed studies, manuscripts posted to preprint servers, expert meta-analyses, and numerous epidemiological analyses of regions with ivermectin distribution campaigns.

Therapeutic Advances: A large majority of randomized and observational controlled trials of ivermectin are reporting repeated, large magnitude improvements in clinical outcomes. Numerous prophylaxis trials demonstrate that regular ivermectin use leads to large reductions in transmission. Multiple, large "natural experiments" occurred in regions that initiated "ivermectin distribution" campaigns followed by tight, reproducible, temporally associated decreases in case counts and case fatality rates compared with nearby regions without such campaigns.

Conclusions: Meta-analyses based on 18 randomized controlled treatment trials of ivermectin in COVID-19 have found large, statistically significant reductions in mortality, time to clinical recovery, and time to viral clearance. Furthermore, results from numerous controlled prophylaxis trials report significantly

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Off-Label Use: This manuscript includes discussion of off-label use in COVID-19 of the FDA-approved medication ivermectin.

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reduced risks of contracting COVID-19 with the regular use of ivermectin. Finally, the many examples of ivermectin distribution campaigns leading to rapid population-wide decreases in morbidity and mortality indicate that an oral agent effective in all phases of COVID-19 has been identified.

Keywords: ivermectin, COVID-19, infectious disease, pulmonary infection, respiratory failure

INTRODUCTION

In early 2020, on the onset of the spreading pandemic, many providers and institutions began to continuously review the rapidly emerging basic science, translational, and clinical data to identify potentially effective treatment options for COVID-19. Although there is now a small and increasing number of therapeutics showing some efficacy in important clinical outcomes, chief of which are corticosteroids in moderate to severe illness, the world continues to suffer from a worsening crisis with the potential of again overwhelming hospitals and intensive care units (ICU). As of February 21, 2020, the number of deaths attributed to COVID-19 in the United States reached 510,248 with more than 9.3 million active cases, the highest number to date. In addition, multiple European countries have imposed new rounds of restrictions and lockdowns.

Further compounding these alarming developments was a wave of recently published results from therapeutic randomized controlled trials conducted on medicines believed effective for COVID-19 that found a lack of impact on mortality in hospitalized patients with the use of remdesivir, hydroxychloroquine, lopinavir/ritonavir, interferon, convalescent plasma, and monoclonal antibody therapy.[1–4] One year into the pandemic, the only therapy considered "proven" as a life-saving treatment in COVID-19 is the use of corticosteroids in patients with moderate to severe illness.[5,6] Similarly, most concerning is the fact that no agent has yet proven effective in outpatients to prevent disease progression to prevent hospitalization.

More recently, trial results of ivermectin, a widely used antiparasitic medicine with known antiviral and anti-inflammatory properties, have been showing benefits in multiple important clinical and virologic outcomes, including mortality. Although growing numbers of the studies supporting this conclusion have passed through peer review, approximately half of the remaining trials data are from manuscripts uploaded to medical preprint servers, a now standard practice for both rapid dissemination and adoption of new therapeutics throughout the pandemic. Following is a comprehensive review of the available efficacy data as of December 12, 2020, taken from in vitro, animal, clinical, and real-world studies all showing the above impacts of ivermectin in COVID-19.

History of ivermectin

In 1975, Professor Satoshi Omura at the Kitsato institute in Japan isolated an unusual *Streptomyces* bacterium from the soil near a golf course along the southeast coast of Honshu, Japan. Omura, along with William Campbell, found that the bacterial culture could cure mice infected with the roundworm *Heligmosomoides polygyrus*. Campbell isolated the active compounds from the bacterial culture, naming them "avermectins" and the bacterium *S. avermitilis* for the compounds' ability to clear mice of worms.[7] Despite decades of searching around the world, the Japanese microorganism remains the only source of avermectin ever found. Ivermectin, a derivative of avermectin, then proved revolutionary. Originally introduced as a veterinary drug, it soon made historic impacts in human health, improving the nutrition, general health, and well-being of billions of people worldwide ever since it was first used to treat onchocerciasis (river blindness) in humans in 1988. It proved ideal in many ways, given that it was highly effective, broad-spectrum, safe, well tolerated, and could be easily administered.[7] Although it was used to treat a variety of internal nematode infections, it was most known as the essential mainstay of 2 global disease elimination campaigns that has nearly eliminated the world of two of its most disfiguring and devastating diseases. The unprecedented partnership between Merck & Co. Inc, and the Kitasato Institute combined with the aid of international health care organizations has been recognized by many experts as one of the greatest medical accomplishments of the 20th century. One example was the decision by Merck & Co to donate ivermectin doses to support the Mectizan Donation Program that then provided more than 570 million treatments in its first 20 years alone.[8] Ivermectin's impacts in controlling onchocerciasis and lymphatic filariasis, diseases which blighted the lives of billions of the poor and disadvantaged throughout the tropics, is why its discoverers were awarded the Nobel Prize in Medicine in 2015 and the reason for its inclusion on the World Health Organization's (WHO) "List of Essential Medicines." Furthermore, it has also been used to successfully overcome several other human diseases and new uses for it are continually being found.[7]

Preclinical studies of Ivermectin's activity against SARS-CoV-2

Since 2012, a growing number of cellular studies have demonstrated that ivermectin has antiviral properties against an increasing number of RNA viruses, including influenza, *Zika*, HIV, *Dengue*, and most importantly, SARS-CoV-2.[9–17] Insights into the mechanisms of action by which ivermectin both interferes with the entrance and replication of SARS-CoV-2 within human cells are mounting. Caly et al[18] first reported that ivermectin significantly inhibits SARS-CoV-2 replication in a cell culture model, observing the near absence of all viral material 48 hours after exposure to ivermectin. However, some questioned whether this observation is generalizable clinically given the inability to achieve similar tissue concentrations used in their experimental model using standard or even massive doses of ivermectin.[19,20] It should be noted that the concentrations required for an effect in cell culture models bear little resemblance to human physiology given the absence of an active immune system working synergistically with a therapeutic agent, such as ivermectin. Furthermore, prolonged durations of exposure to a drug likely would require a fraction of the dosing in short-term cell model exposure. Furthermore, multiple coexisting or alternate mechanisms of action likely explain the clinical effects observed, such as the competitive binding of ivermectin with the host receptor-binding region of SARS-CoV-2 spike protein, as proposed in 6 molecular modeling studies.[21–26] In 4 of the studies, ivermectin was identified as having the highest or among the highest of binding affinities to spike protein S1 binding domains of SARS-CoV-2 among hundreds of molecules collectively examined, with ivermectin not being the particular focus of study in 4 of these studies.[27] This is the same mechanism by which viral antibodies, in particular, those generated by the Pfizer and Moderna vaccines contain the SARS-CoV-2 virus. The high binding activity of ivermectin to the SARS-CoV-2 spike protein could limit binding to either the ACE-2 receptor or sialic acid receptors, respectively, either preventing cellular entry of the virus or preventing hemagglutination, a recently proposed pathologic mechanism in COVID-19.[21,22,26–28] Ivermectin has also been shown to bind to or interfere with multiple essential structural and nonstructural proteins required by the virus to replicate.[26,29] Finally, ivermectin also binds to the SARS-CoV-2 RNA-dependent RNA polymerase (RdRp), thereby inhibiting viral replication.[30]

Arevalo et al investigated in a murine model infected with a type 2 family RNA coronavirus similar to SARS-CoV-2, (mouse hepatitis virus), the response to 500 μg/kg of ivermectin versus placebo.[31] The study included 40 infected mice, with 20 treated with ivermectin, 20 with phosphate-buffered saline, and then 16 uninfected control mice that were also given phosphate-buffered saline. At day 5, all the mice were killed to obtain tissues for examination and viral load assessment. The 20 nonivermectin-treated infected mice all showed severe hepatocellular necrosis surrounded by a severe lymphoplasmacytic inflammatory infiltration associated with a high hepatic viral load (52,158), whereas in the ivermectin-treated mice a much lower viral load was measured (23,192; $P < 0.05$), with only few livers in the ivermectin-treated mice showing histopathological damage such that the differences between the livers from the uninfected control mice were not statistically significant.

Dias De Melo et al[32] recently posted the results of a study they did with golden hamsters that were intranasally inoculated with SARS-CoV-2 virus, and at the time of the infection, the animals also received a single subcutaneous injection of ivermectin at a dose of 0.4 mg/kg on day 1. Control animals received only the physiologic solution. They found the following among the ivermectin-treated hamsters: a dramatic reduction in anosmia (33.3% vs. 83.3%, $P = 0.03$), which was also sex dependent in that the male hamsters exhibited a reduction in clinical score while the treated female hamsters failed to show any sign of anosmia. They also found significant reductions in cytokine concentrations in the nasal turbinates and lungs of the treated animals, despite the lack of apparent differences in viral titers.

Despite these mounting insights into the existing and potential mechanisms of action of ivermectin both as a prophylactic and treatment agent, it must be emphasized that significant research gaps remain and that many further in vitro and animal studies should be undertaken to better define not only these mechanisms but also to further support ivermectin's role as a prophylactic agent, especially in the optimal dose and frequency required.

Preclinical studies of ivermectin's anti-inflammatory properties

Given that little viral replication occurs in the later phases of COVID-19, nor can virus be cultured, and only in a minority of autopsies can viral cytopathic changes be found,[33–35] the most likely pathophysiologic mechanism is that identified by Li et al[36] where they showed that the nonviable RNA fragments of SARS-CoV-2 lead to a high mortality and morbidity in COVID-19 through the provocation of an overwhelming and injurious inflammatory response. Based on these insights and the clinical benefits of ivermectin in the late phase of disease to be reviewed below, it seems that the increasingly well-described in vitro properties of ivermectin as an inhibitor of inflammation are far more clinically potent than previously recognized. The growing list of studies demonstrating the anti-inflammatory properties of ivermectin include its

ability to inhibit cytokine production after lipopolysaccharide exposure, downregulate transcription of NF-kB, and limit the production of both nitric oxide and prostaglandin E_2.[37–39]

Exposure prophylaxis studies of ivermectin's ability to prevent transmission of COVID-19

Data are also now available showing large and statistically significant decreases in the transmission of COVID-19 among human subjects based on data from 3 randomized controlled trials (RCTs) and 5 observational controlled trials (OCTs) with 4 of the 8 (2 of them RCTs) published in peer-reviewed journals.[40–46]

Elgazzar and colleagues[45] at Benha University in Egypt randomized 200 health care and household contacts of patients with COVID-19 where the intervention group consisted of 100 patients given a high dose of 0.4 mg/kg on day 1 and a second dose on day 7 in addition to wearing personal protective equipment, whereas the control group of 100 contacts wore personal protective equipment alone. They reported a large and statistically significant reduction in contacts testing positive by Reverse Transcriptase Polymerase Chain Reaction (PCR) when treated with ivermectin versus controls, 2% versus 10%, $P < 0.05$.

Shouman conducted an RCT at Zagazig University in Egypt, including 340 (228 treated and 112 control) family members of patients positive for SARS-CoV-2 through PCR.[44] Ivermectin (approximately 0.25 mg/kg) was administered twice, on the day of the positive test and 72 hours later. After a two-week follow-up, a large and statistically significant decrease in COVID-19 symptoms among household members treated with ivermectin was found, 7.4% versus 58.4%, $P < 0.001$.

Recently, Alam et al from Bangladesh performed a prospective observational study of 118 patients who were evenly split into those who volunteered for either the treatment or control arms, described as a persuasive approach. Although this method, along with the study being unblinded, likely led to confounders, the difference between the 2 groups was so large (6.7% vs. 73.3%, $P < 0.001$) and similar to the other prophylaxis trial results that confounders alone are unlikely to explain such a result.[47] Carvallo et al also performed a prospective observational trial where they gave healthy volunteers ivermectin and carrageenan daily for 28 days and matched them to similarly healthy controls who did not take the medicines.[40] Of the 229 study subjects, 131 were treated with 0.2 mg of ivermectin drops taken by mouth 5 times per day. After 28 days, none of those receiving ivermectin in the prophylaxis group had tested positive for SARS-COV-2 versus 11.2% of patients in the control arm ($P < 0.001$). In a much larger follow-up prospective, observational controlled trial by the same group that included 1195 health care workers, they found that over a 3-month period there were no infections recorded among the 788 workers who took weekly ivermectin prophylaxis, whereas 58% of the 407 controls had become ill with COVID-19. This study demonstrates that remarkable protection against transmission can be achieved among high-risk health care workers by taking 12 mg once weekly.[40] The Carvallo IVERCAR protocol was also separately tested in a prospective RCT by the Health Ministry of Tucuman, Argentina, where they found that among 234 health care workers, the intervention group that took 12 mg once weekly, only 3.4% contracted COVID-19 versus 21.4% of controls, $P < .0001$.[46]

The need for weekly dosing in the Carvallo study over a 4-month period may not have been necessary given that, in a recent RCT from Dhaka, Bangladesh, the intervention group (n = 58) took 12 mg once monthly for a similar 4-month period and also reported a large and statistically significant decrease in infections compared with controls, 6.9% versus 73.3%, $P < 0.05$.[47] Then, in a large retrospective observational case–control study from India, Behera et al[41] reported that among 186 case–control pairs (n = 372) of health care workers, they identified 169 participants who had taken some form of prophylaxis, with 115 participants that had taken ivermectin. After matched pair analysis, they reported that in the workers who had taken 2 dose ivermectin prophylaxis, the odds ratio for contracting COVID-19 was markedly decreased (0.27, 95% confidence interval (CI) 0.15–0.51). Notably, one dose prophylaxis was not found to be protective in this study. Based on both their study finding and the Egyptian prophylaxis study, the All India Institute of Medical Sciences instituted a prophylaxis protocol for their health care workers where they now take two 0.3 mg/kg doses of ivermectin 72 hours apart and repeat the dose monthly.

Data that further illuminates the potential protective role of ivermectin against COVID-19 come from a study of nursing home residents in France which reported that in a facility that suffered a scabies outbreak where all 69 residents and 52 staff were treated with ivermectin,[41] they found that during the period surrounding this event, 7 of the 69 residents fell ill with COVID-19 (10.1%). In this group with an average age of 90 years, only one resident required oxygen support and no resident died. In a matched control group of residents from surrounding facilities, they found 22.6% of residents fell ill and 4.9% died.

Further evidence supporting the efficacy of ivermectin as a prophylaxis agent was published recently in the *International Journal of Antimicrobial agents* where a group of researchers analyzed data using the prophylactic chemotherapy databank administered by the WHO along with case counts obtained by Worldometers, a public data

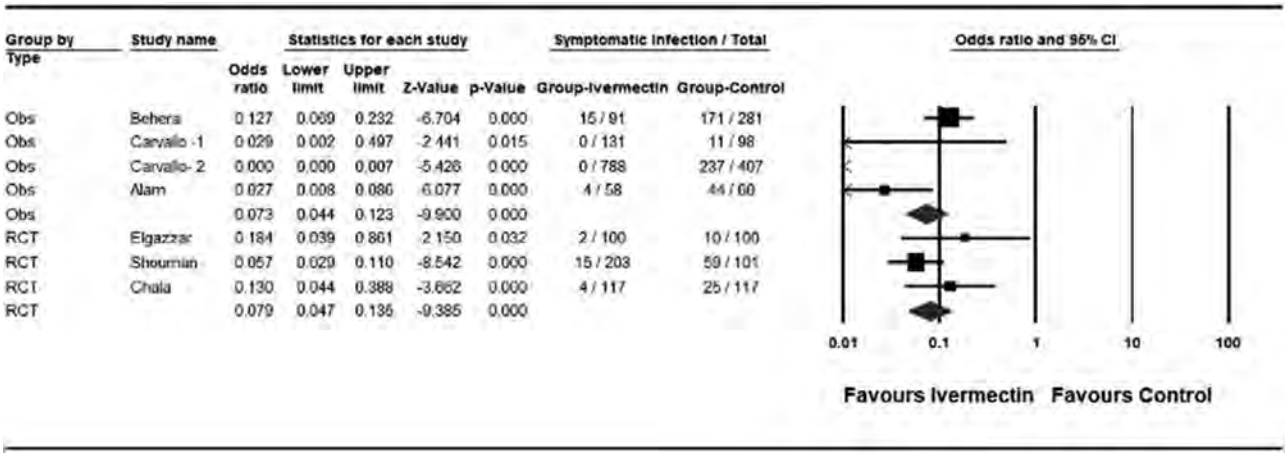

FIGURE 1. Meta-analysis of ivermectin prophylaxis trials in COVID-19. OBS, observational study; RCT, randomized controlled trial. Symbols: Squares: Indicate treatment effect of an individual study. Large diamond: Reflect summary of study design immediately above. Size of each symbol correlates with the size of the confidence interval around the point estimate of treatment effect with larger sizes indicating a more precise confidence interval.

aggregation site used by among others, the Johns Hopkins University.[42] When they compared the data from countries with active ivermectin mass drug administration programs for the prevention of parasite infections, they discovered that the COVID-19 case counts were significantly lower in the countries with recently active programs, to a high degree of statistical significance, $P < 0.001$.

Figure 1 presents a meta-analysis performed by the study authors of the controlled ivermectin prophylaxis trials in COVID-19.

Further data supporting a role of ivermectin in decreasing transmission rates can be found from South American countries where, in retrospect, large "natural experiments" seem to have occurred. For instance, beginning as early as May, various regional health ministries and governmental authorities within Peru, Brazil, and Paraguay initiated "ivermectin distribution" campaigns to their citizen populations.[48] In one such example from Brazil, the cities of Itajai, Macapa, and Natal distributed massive amounts of ivermectin doses to their city's population, where in the case of Natal, 1 million doses were distributed. The distribution campaign of Itajai began in mid-July, in Natal they began on June 30th, and in Macapa, the capital city of Amapa and others nearby, they incorporated ivermectin into their treatment protocols in late May after they were particularly hard hit in April. The data in Table 1 were obtained from the official Brazilian government site and the national press consortium and show large decreases in case counts in the 3 cities soon after distribution began compared with their neighboring cities without such campaigns.

The decreases in case counts among the 3 Brazilian cities given in Table 1 were also associated with reduced mortality rates as summarized in Table 2.

Clinical studies on the efficacy of ivermectin in treating mildly ill outpatients

Currently, 7 trials that include a total of more than 3000 patients with mild outpatient illness have been completed, a set composed of 7 RCTs and 4 case series.[49–60]

Table 1. Comparison of case count decreases among Brazilian cities with and without ivermectin distribution campaigns.

Region	New cases	June	July	August	Population 2020 (1000)	% Decline in new cases between June and August 2020
South	**Itajaí**	**2123**	**2854**	**998**	**223**	**−53%**
	Chapecó	1760	1754	1405	224	−20%
North	**Macapá**	**7966**	**2481**	**2370**	**503**	**−70%**
	Ananindeua	1520	1521	1014	535	−30%
North East	**Natal**	**9009**	**7554**	**1590**	**890**	**−82%**
	João Pessoa	9437	7963	5384	817	−43%

Bolded cities distributed ivermectin, neighboring regional city below did not.

Table 2. Change in death rates among neighboring regions in Brazil.

Region	State	% Change in average deaths/week compared with 2 weeks before
South	**Santa Catarina**	**–36%**
	PARANÁ	–3%
	Rio Grande do Sul	–5%
North	**Amapá**	**–75%**
	AMAZONAS	–42%
	Pará	+13%
North East	**Rio Grande do Norte**	**–65%**
	CEARÁ	+62%
	Paraíba	–30%

Bolded regions contained a major city that distributed ivermectin to its citizens, the other regions did not.

The largest, a double-blinded RCT by Mahmud[49] was conducted in Dhaka, Bangladesh, and targeted 400 patients with 363 patients completing the study. In this study, as in many other of the clinical studies to be reviewed, either a tetracycline (doxycycline) or macrolide antibiotic (azithromycin) was included as part of the treatment. The importance of including antibiotics such as doxycycline or azithromycin is unclear; however, both tetracycline and macrolide antibiotics have recognized anti-inflammatory, immunomodulatory, and even antiviral effects (58–61). Although the posted data from this study does not specify the amount of mildly ill outpatients versus hospitalized patients treated, important clinical outcomes were profoundly affected, with increased rates of early improvement (60.7% vs. 44.4% $P < 0.03$) and decreased rates of clinical deterioration (8.7% vs. 17.8%, $P < 0.02$). Given that mildly ill outpatients mainly comprised the study cohort, only 2 deaths were observed (both in the control group).

Ravikirti performed a double-blinded RCT of 115 patients, and although the primary outcome of PCR positivity on day 6 was no different, the secondary outcome of mortality was 0% versus 6.9%, $P = .019$.[60] Babalola in Nigeria also performed a double-blinded RCT of 62 patients, and in contrast to Ravikirti, they found a significant difference in viral clearance between both the low-dose and high-dose treatment groups and controls in a dose dependent fashion, $P = .006$.[59]

Another RCT by Hashim et al[53] in Baghdad, Iraq, included 140 patients equally divided; the control group received standard care, and the treated group included a combination of both outpatient and hospitalized patients. In the 96 patients with mild-to-moderate outpatient illness, they treated 48 patients with a combination of ivermectin/doxycycline and standard of care and compared outcomes with the 48 patients treated with standard of care alone. The standard of care in this trial included medicines such as dexamethasone 6 mg/d or methylprednisolone 40 mg twice per day if needed, vitamin C 1000 mg twice/day, zinc 75–125 mg/d, vitamin D3 5000 IU/day, azithromycin 250 mg/d for 5 days, and acetaminophen 500 mg as needed. Although no patients in either group progressed or died, the time to recovery was significantly shorter in the ivermectin-treated group (6.3 days vs. 13.7 days, $P < 0.0001$).

Chaccour et al conducted a small, double-blinded RCT in Spain where they randomized 24 patients to ivermectin versus placebo, and although they found no difference in PCR positivity at day 7, they did find statistically significant decreases in viral loads, patient days of anosmia (76 vs. 158, $P < 0.05$), and patient days with cough (68 vs. 98, $P < 0.05$).[57]

Another RCT of ivermectin treatment in 116 outpatients was performed by Chowdhury et al in Bangladesh where they compared a group of 60 patients treated with the combination of ivermectin/doxycycline to a group of 60 patients treated with hydroxychloroquine/doxycycline with a primary outcome of time to negative PCR.[54] Although they found no difference in this outcome, in the treatment group, the time to symptomatic recovery approached statistical significance (5.9 days vs. 7.0 days, $P = 0.07$). In another smaller RCT of 62 patients by Podder et al, they also found a shorter time to symptomatic recovery that approached statistical significance (10.1 days vs. 11.5 days, $P > 0.05$, 95% CI, 0.86–3.67).[55]

A medical group in the Dominican Republic reported a case series of 2688 consecutive symptomatic outpatients seeking treatment in the emergency department, most whom were diagnosed using a clinical algorithm. The patients were treated with a high-dose ivermectin of 0.4 mg/kg for one dose along with 5 days of azithromycin. Remarkably, only 16 of the 2688 patients (0.59%) required subsequent hospitalization with only a single death recorded.[61]

In another case series of 100 patients in Bangladesh, all treated with a combination of 0.2 mg/kg ivermectin and doxycycline, they found that no patient required hospitalization nor died, and all patients' symptoms improved within 72 hours.[62]

A case series from Argentina reported on a combination protocol that used ivermectin, aspirin, dexamethasone, and enoxaparin. In the 135 mild illness patients, all survived.[50] Similarly, a case series from Mexico of 28 consecutively treated patients with ivermectin, all were reported to have recovered with an average time to full recovery of only 3.6 days.[58]

Clinical studies of the efficacy of ivermectin in hospitalized patients

Studies of ivermectin among more severely ill hospitalized patients include 6 RCTs, 5 OCTs, and a database analysis study.[45,51–53,63–70]

The largest RCT in hospitalized patients was performed concurrent with the prophylaxis study reviewed above by Elgazzar et al.[45] Four hundred patients were randomized among 4 treatment groups of 100 patients each. Groups 1 and 2 included mild/moderate illness patients alone, with group 1 treated with one dose 0.4 mg/kg ivermectin plus standard of care (SOC) and group 2 received hydroxychloroquine 400 mg twice on day 1 then 200 mg twice daily for 5 days plus standard of care. There was a statistically significant lower rate of progression in the ivermectin-treated group (1% vs. 22%, $P < 0.001$), with no deaths and 4 deaths, respectively. Groups 3 and 4 included only severely ill patients, with group 3 again treated with a single dose of 0.4 mg/kg plus SOC, whereas group 4 received hydroxychloroquine plus SOC. In this severely ill subgroup, the differences in outcomes were even larger, with lower rates of progression 4% versus 30% and mortality 2% versus 20% ($P < 0.001$).

The one largely outpatient RCT conducted by Hashim reviewed above also included 22 hospitalized patients in each group. In the ivermectin/doxycycline-treated group, there were 11 severely ill patients and 11 critically ill patients, whereas in the standard of care group, only severely ill patients (n = 22) were included because of their ethical concerns of including critically ill patients in the control group (*45*). This decision led to a marked imbalance in the severity of illness between these hospitalized patient groups. However, despite the mismatched severity of illness between groups and the small number of patients included, beneficial differences in outcomes were seen, but not all reached statistical significance. For instance, there was a large reduction in the rate of progression of illness (9% vs. 31.8%, $P = 0.15$) and, most importantly, there was a large difference in mortality among the severely ill groups that reached a borderline statistical significance (0% vs. 27.3%, $P = 0.052$). Another important finding was the relatively low mortality rate of 18% found among the subset of critically ill patients, all of whom were treated with ivermectin.

A recent RCT from Iran found a dramatic reduction in mortality with ivermectin use.[65] Among multiple ivermectin treatment arms (different ivermectin dosing strategies were used in the intervention arms), the average mortality was reported as 3.3%, whereas the average mortality within the standard care and placebo arms was 18.8%, with an odds ratio (OR) of 0.18 (95% CI 0.06–0.55, $P < 0.05$).

Spoorthi[64] and Sasanak performed a prospective trial of 100 hospitalized patients whereby they treated 50 with ivermectin and doxycycline, whereas the 50 controls were given a placebo consisting of vitamin B6. Although no deaths were reported in either group, the ivermectin treatment group had a statistically significant shorter hospital length of stay (LOS) 3.7 days versus 4.7 days, $P = 0.03$, and shorter time to complete resolution of symptoms, 6.7 days versus 7.9 days, $P = 0.01$.

The largest OCT (n = 280) in hospitalized patients was conducted by Rajter et al at Broward Health Hospitals in Florida and was recently published in the major medical journal *Chest* (*43*). They performed a retrospective OCT using a propensity-matched design on 280 consecutive treated patients and compared those treated with ivermectin to those without. One hundred seventy-three patients were treated with ivermectin (160 received a single dose and 13 received a second dose at day 7) while 107 were not.[63] In both unmatched and propensity-matched cohort comparisons, similar, large, and statistically significant lower mortality was found among ivermectin-treated patients (15.0% vs. 25.2%, $P = 0.03$). Furthermore, in the subgroup of patients with severe pulmonary involvement, mortality was profoundly reduced when treated with ivermectin (38.8% vs. 80.7%, $P = 0.001$).

Another large OCT in Bangladesh compared 115 patients treated with ivermectin to a standard care cohort consisting of 133 patients.[51] Despite a significantly higher proportion of patients in the ivermectin group being men (ie, with well-described, lower survival rates in COVID), the groups were otherwise well matched, yet the mortality decrease was statistically significant (0.9% vs. 6.8%, $P < 0.05$). The largest OCT is a study from Brazil, published as a letter to the editor and included almost 1500 patients.[66] Although the primary data were not provided, they reported that in 704 hospitalized patients treated with a single dose of 0.15 mg/kg ivermectin, compared with 704 controls, overall mortality was reduced (1.4% vs. 8.5%, HR 0.2, 95% CI 0.12–0.37, $P < 0.0001$). Similarly, in the patients on mechanical ventilation, mortality was also reduced (1.3% vs. 7.3%). A small study from Baghdad, Iraq, compared 16 ivermectin-treated patients with 71

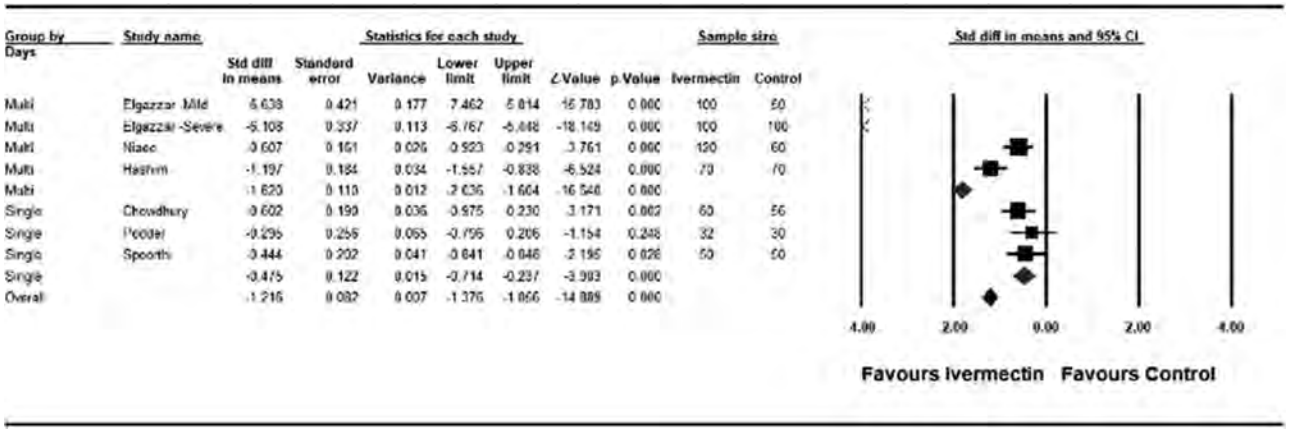

FIGURE 2. Meta-analysis of the outcome of time to clinical recovery from controlled trials of ivermectin treatment in COVID-19. OBS, observational study; RCT, randomized controlled trial. Symbols: Squares: Indicate treatment effect of an individual study. Large diamond: Reflect summary of study design immediately above. Small diamond: Sum effect of all trial designs. Size of each symbol correlates with the size of the confidence interval around the point estimate of treatment effect with larger sizes indicating a more precise confidence interval.

controls.[52] This study also reported a significant reduction in length of hospital stay (7.6 days vs. 13.2 days, $P < 0.001$) in the ivermectin group. In a study reporting on the first 1000 patients treated in a hospital in India, they found that in the 34 patients treated with ivermectin alone, all recovered and were discharged, whereas in more than 900 patients treated with other agents, there was an overall mortality of 11.1%.[70]

Meta-analyses of the above controlled treatment trials were performed by the study authors focused on the 2 important clinical outcomes: time to clinical recovery and mortality (Figures 2 and 3). The consistent and reproducible signals leading to large overall statistically significant benefits from within both study designs are remarkable, especially given that in several of the studies treatment was initiated late in the disease course.

Details of the prophylaxis, early, and late treatment trials of ivermectin in COVID-19 can be found in Table 3.

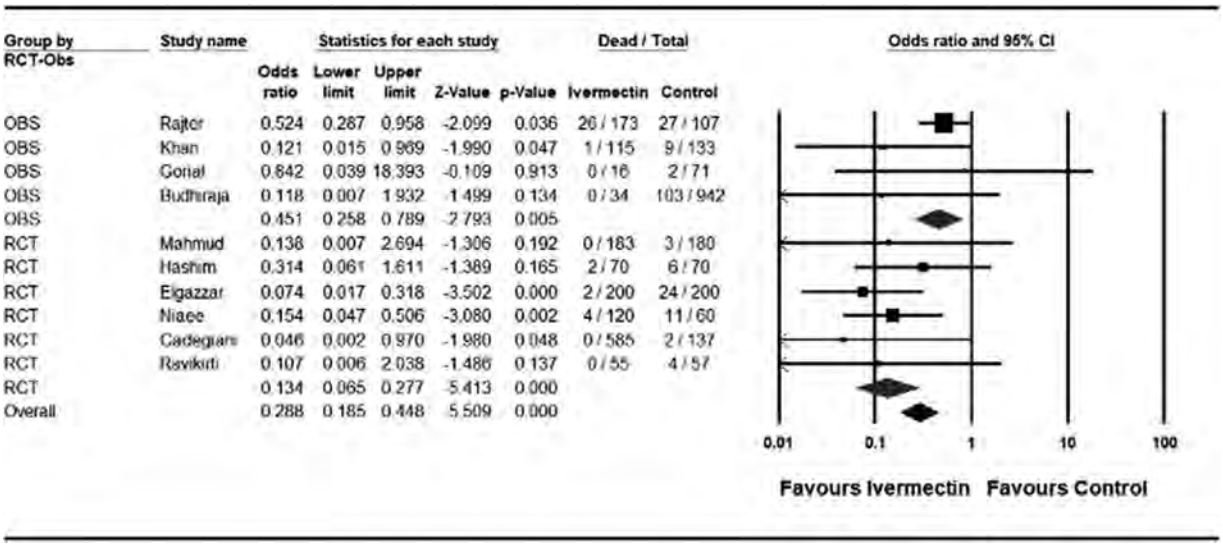

FIGURE 3. Meta-analysis of the outcome of mortality from controlled trials of ivermectin treatment in COVID-19. OBS, observational study; RCT, randomized controlled trial. Symbols: Squares: Indicate treatment effect of an individual study. Large diamond: Reflect summary of study design immediately above. Small diamond: Sum effect of all trial designs. Size of each symbol correlates with the size of the confidence interval around the point estimate of treatment effect with larger sizes indicating a more precise confidence interval.

Table 3. Clinical studies assessing the efficacy of ivermectin in the prophylaxis and treatment of COVID-19.

Prophylaxis Trials Author, Country, source	Study design, size	Study subjects	Ivermectin dose	Dose frequency	Clinical outcomes reported
Prophylaxis trials					
Shouman W, Egypt *www.clinicaltrials.gov* NCT04422561	RCT N = 340	Household members of pts with +COVID-19 PCR test	40–60 kg: 15 mg, 60–80 kg: 18 mg, and > 80 kg: 24 mg	Two doses, 72 hours apart	7.4% versus 58.4% developed COVID-19 symptoms, $P < 0.001$
Elgazzar A, Egypt ResearchSquare doi.org/10.21203/rs.3.rs-100956/v1	RCT N = 200	Health care and household contacts of pts with +COVID-19 PCR test	0.4 mg/kg	Two doses, day 1 and day 7	2% versus 10% tested positive for COVID-19 $P < 0.05$
Chala R, Argentina NCT04701710 *Clinicaltrials.gov*	RCT N = 234	Health care workers	12 mg	Every 7 d	3.4% versus 21.4%, $P = 0.0001$.
Carvallo H, Argentina *Journal of Biochemical Research and Investigation* doi.org/10.31546/2633–8653.1007	OCT N = 229	Healthy patients negative for COVID-19 PCR test	0.2 mg drops	1 drop 5 times a d x 28 d	0.0% versus 11.2% contracted COVID-19 $P < 0.001$
Alam MT, Bangladesh *European J Med Hlth Sciences* 10.24018/ejmed.2020.2.6.599	OCT N = 118	Health care workers	12 mg	Monthly	6.9% versus 73.3%, $P < 0.05$
Carvallo H, Argentina *Journal of Biochemical Research and Investigation* doi.org/10.31546/2633–8653.1007	OCT N = 1195	Health care workers	12 mg	Once weekly for up to 10 wk	0.0% of the 788 workers taking ivermectin versus 58% of the 407 controls contracted COVID-19.
Behera P, India *medRxiv* doi.org/10.1101/2020.10.29.20222661	OCT N = 186 case control pairs	Health care workers	0.3 mg/kg	Day 1 and day 4	2 doses reduced odds of contracting COVID-19 (OR 0.27 95% CI 0.16–0.53)
Bernigaud C, France *Annales de Dermatologie et de Venereologi* doi.org/10.1016/j.annder.2020.09.231	OCT N = 69 case control pairs	Nursing home residents	0.2 mg/kg	Once	10.1% versus 22.6% residents contracted COVID-19 0.0% versus 4.9% mortality
Hellwig M, USA *J Antimicrobial Agents* doi.org/10.1016/j.ijantimicag.2020.106,248	OCT N = 52 countries	Countries with and without IVM prophylaxis programs	Unknown	Variable	Significantly lower-case incidence of COVID-19 in African countries with IVM prophylaxis programs $P < 0.001$

Clinical trials–Outpatients % Ivermectin versus % Controls

Prophylaxis Trials Author, Country, source	Study design, size	Study subjects	Ivermectin dose	Dose frequency	Clinical outcomes reported
Mahmud R, Bangladesh *www.clinicaltrials.gov* NCT0452383	DB-RCT N = 363	Outpatients and hospitalized	12 mg + doxycycline	Once, within 3 days of PCR+ test	Early improvement 60.7% versus 44.4%, $P < 0.03$, deterioration 8.7% versus 17.8%, $P < 0.02$
Chowdhury A, Bangladesh *Research Square* doi.org/10.21203/rs.3.rs-38896/v1	RCT N = 116	Outpatients	0.2 mg//kg + doxycycline	Once	Recovery time 5.9 versus 9.3 days ($P = 0.07$)
Ravikirti, India *medRxiv* doi.org/10.1101/2021.01.05.21249310	DB-RCT N = 115	Mild–moderate illness	12 mg	Daily for 2 d	No diff in day 6 PCR + 0% versus 6.9% mortality, $P = 0.019$
Babalola OE, Nigeria medRxiv doi.org/10.1101/2021.01.05.21249131	DB-RCT N = 62	Mild–moderate illness	6 mg and 12 mg	Every 48 hours × 2 wk	Time to viral clearance: 4.6 days high dose versus 6.0 days low dose versus 9.1 days control ($P = 0.006$)
Podder CS, Bangladesh *IMC J Med Sci 2020;14(2)*	RCT N = 62	Outpatients	0.2 mg/kg	Once	Recovery time 10.1 versus 11.5 days (NS), average time 5.3 versus 6.3 (NS)
Chaccour C. Spain *Research Square* doi.org/10.21203/rs.3.rs-116547/v1	DB-RCT N = 24	Outpatients	0.4 mg/kg	Once	No diff in PCR+ day 7, lower viral load d 4 and 7, ($P < 0.05$), 76 versus 158 pts. d of anosmia ($P < 0.05$), 68 versus 98 pts. d of cough ($P < 0.05$)
Morgenstern J, Dominican Republic *medRxiv* doi.org/10.1101/2020.10.29.20222505	Case series N = 3099	Outpatients and hospitalized	Outpatients: 0.4 mg/kg hospital patients: 0.3 mg/kg	Outpatients: 0.3 mg/kg × 1 dose Inpatients: 0.3 mg/kg, days 1,2,6, and 7	Mortality = 0.03% in 2688 outpatients, 1% in 300 non-ICU hospital patients, and 30.6% in 111 ICU patients
Carvallo H, Argentina *medRxiv* doi.org/10.1101/2020.09.10.20191619	Case series N = 167	Outpatients and hospitalized	24 mg = mild, 36 mg = moderate, and 48 mg = severe	Days 0 and 7	All 135 with mild illness survived, 1/32 (3.1% of hospitalized) patients died
Alam A, Banglades *J of Bangladesh College Phys and Surg*, 2020; 38:10-15 doi.org/10.3329/jbcps.v38i0.47512	Case series N = 100	Outpatients	0.2 mg/kg/kg + doxycycline	Once	All improved within 72 h

(*Continued on next page*)

Table 3. (*Continued*) Clinical studies assessing the efficacy of ivermectin in the prophylaxis and treatment of COVID-19.

Clinical trials–Outpatients					% Ivermectin versus % Controls
Prophylaxis Trials Author, Country, source	Study design, size	Study subjects	Ivermectin dose	Dose frequency	Clinical outcomes reported
Espatia-Hernandez G, Mexico *Biomedical Research* www.biomedres.info/biomedi..-proof-of-concept-study-14435.html	Case series N = 28	Outpatients	6 mg	Days 1,2, 7, and 8	All pts recovered average recovery time 3.6 d

Clinical trials–Hospitalized patients					% Ivermectin versus % Controls
Prophylaxis Trials Author, Country, source	Study design, size	Study subjects	Ivermectin dose	Dose frequency	Clinical outcomes reported
Elgazzar A, Egypt ResearchSquare doi.org/10.21203/rs.3.rs-100956/v1	OL-RCT N = 400	Hospitalized patients	0.4 mg/kg	Daily for 4 days	Moderately ill: worsened 1% versus 22%, $P<0.001$. Severely ill: worsened 4% versus 30% mortality 2% versus 20% both with $P < 0.001$
Niaee S. M, Research Square doi.org/10.21203/rs.3.rs-109670/v1	DB-RCT N = 180	Hospitalized patients	0.2, 0.3, and 0.4 mg/kg (3 dosing strategies)	Once versus Days 1,3,5	Mortality 3.3% versus 18.3%. OR 0.18, (0.06–0.55, $P < 0.05$)
Hashim H, Iraq medRxiv doi.org/10.1101/2020.10.26.20219345	SB-RCT N=140	2/3 outpatients and 1/3 hospital pts	0.2 mg/kg + doxycycline	Daily for 2–3 d	Recovery time 6.3 versus 13.6 days ($P<0.001$), 0% versus 27.3% mortality in severely ill ($P = 0.052$)
Spoorthi S, India AIAM, 2020; 7(10):177-182	PCT N = 100	Hospitalized patients	0.2 mg/kg+ doxycycline	Once	Shorter hospital LOS, 3.7 versus 4.7 days, $P = 0.03$, faster resolution of symptoms, 6.7 versus 7.9 days, $P = 0.01$
Ahmed S. Dhaka, Bangladesh International journal of Infectious disease doi.org/10.1016/j.ijid.2020.11.191	DB-RCT N = 72	Hospitalized patients	12 mg	Daily for 5 d	Faster viral clearance 9.7 versus 12.7 days, $P = 0.02$
Chachar AZK, Pakistan Int J Sciences doi.org/10.18483/ijSci.2378	DB-RCT N = 50	Hospitalized patients-mild	12 mg	Two doses day 1 and one dose day 2	64% versus 60% asymptomatic by day 7
Portman-Baracco A, Brazil	OCT		0.15 mg/kg	Once	

(*Continued on next page*)

Table 3. (*Continued*) Clinical studies assessing the efficacy of ivermectin in the prophylaxis and treatment of COVID-19.

Clinical trials–Hospitalized patients					% Ivermectin versus % Controls
Prophylaxis Trials Author, Country, source	Study design, size	Study subjects	Ivermectin dose	Dose frequency	Clinical outcomes reported
Arch Bronconeumol. 2020 doi.org/10.1016/j.arbres.2020.06.011	N = 1408	Hospitalized patients			Overall mortality 1.4% versus 8.5%, HR 0.2, 95% CI 0.12–0.37, $P < 0.0001$
Rajter JC, Florida Chest 2020 doi.org/10.1016/j.chest.2020.10.009	OCT N=280	Hospitalized patients	0.2 mg/kg + azithromycin	Day 1 and day 7 if needed	Overall mortality 15.0% versus 25.2%, $P = 0.03$, severe illness mortality 38.8% versus 80.7%, $P = 0.001$
Khan X, Bangladesh Arch Bronconeumol. 2020 doi.org/10.1016/j.arbres.2020.08.007	OCT N = 248	Hospitalized patients	12 mg	Once on admission	Mortality 0.9% versus 6.8%, $P < 0.05$, LOS 9 versus 15 days, $P < 0.001$
Gorial FI, Iraq medRxiv doi.org/10.1101/2020.07.07.20145979	OCT N = 87	Hospitalized patients	0.2 mg/kg + HCQ and azithromycin	Once on admission	LOS 7.6 versus 13.2 days, $P < 0.001$, 0/15 versus 2/71 died
Budiraja S. India medRxiv doi.org/10.1101/2020.11.16.20232223	OCT N = 1000 IVM=34	Hospitalized patients	n/a	n/a	100% IVM pts recovered 11.1% mortality in non-IVM-treated pts

DB-RCT, double-blinded randomized controlled trial; HCQ, hydroxychloroquine; IVM, ivermectin; LOS, length of stay; NS, nonstatistically significant, $P > .05$; OCT, observational controlled trial; OL, open label; PCR, polymerase chain reaction; RCT, randomized controlled trial; SB-RCT, single blinded randomized controlled trial.

Ivermectin in post-COVID-19 syndrome

Increasing reports of persistent, vexing, and even disabling symptoms after recovery from acute COVID-19 have been reported and that many have termed the condition as "Long COVID" and patients as "long haulers," estimated to occur in approximately 10%–30% of cases.[71–73] Generally considered as a postviral syndrome consisting of a chronic and sometimes disabling constellation of symptoms which include, in order, fatigue, shortness of breath, joint pains, and chest pain. Many patients describe their most disabling symptom as impaired memory and concentration, often with extreme fatigue, described as "brain fog," and is highly suggestive of the condition myalgic encephalomyelitis/chronic fatigue syndrome, a condition well reported to begin after viral infections, in particular with Epstein–Barr virus. Although no specific treatments have been identified for Long COVID, a recent manuscript by Aguirre-Chang et al from the National University of San Marcos in Peru reported on their experience with ivermectin in such patients.[74] They treated 33 patients who were between 4 and 12 weeks from the onset of symptoms with escalating doses of ivermectin; 0.2 mg/kg for 2 days if mild and 0.4 mg/kg for 2 days if moderate, with doses extended if symptoms persisted. They found that in 87.9% of the patients, resolution of all symptoms was observed after 2 doses with an additional 7% reporting complete resolution after additional doses. Their experience suggests the need for controlled studies to better test efficacy in this vexing syndrome.

Epidemiological data showing impacts of widespread ivermectin use on population case counts and case fatality rates

Similar to the individual cities in Brazil that measured large decreases in case counts soon after distributing ivermectin in comparison to neighboring cities without such campaigns, in Peru, the government approved the use of ivermectin by decree on May 8, 2020, solely based on the in vitro study by Caly et al from Australia.[48] Soon after, multiple state health ministries initiated ivermectin distribution campaigns in an effort to decrease what was at that time some of the highest COVID-19 morbidity and mortality rates in the world. Juan Chamie,[48] a data analyst and member of the FLCCC Alliance, recently posted an article based on 2 critical sets of data that he compiled and compared; first, he identified the timing and magnitude of each region's ivermectin interventions through a review of official communications, press releases, and the Peruvian Situation Room database to confirm the dates of effective delivery, and second, he extracted data on the total all-cause deaths from the region along with COVID-19 case counts in selected age groups over time from the registry of the National Computer System of Deaths (SINADEF) and from the National Institute of Statistics and Informatics.[48] It should be noted that he restricted his analyses to only those citizens older than 60 years to avoid the confounding of rises in the numbers of infected younger patients. With these data, he was then able to compare the timing of major decreases in this age group of both total COVID-19 cases and total excess deaths per 1000,000 people among 8 states in Peru with the initiation dates of their respective ivermectin distribution campaigns as shown in Figure 4.

Figure 5 from the same study presents data on the case fatality rates in patients older than 60 years, again among the 8 states in Peru. Note the dramatically decreased case fatality rates among older patients diagnosed with COVID-19 after ivermectin became widely distributed in those areas, a result which cannot be explained by changes in mask-wearing or lock-downs.

In an even more telling example, Chamie compared the case counts and fatality rates of the 8 states above with the city of Lima, where ivermectin was not distributed nor widely used in treatment during the same period. Figure 6 compares the lack of significant or sustained reductions in case counts or fatalities in Lima with the dramatic reductions in both outcomes among the 8 states with widespread ivermectin distribution.

Another example can be seen from the data compiled from Paraguay, again by Chamie who noted that the government of the state of Alto Parana had launched an ivermectin distribution campaign in early September. Although the campaign was officially described as a "deworming" program, this was interpreted as a guise by the regions' governor to avoid reprimand or conflict with the National Ministry of Health that recommended against the use of ivermectin to treat COVID-19 in Paraguay. The program began with a distribution of 30,000 boxes of ivermectin, and by October 15, the governor declared that there were very few cases left in the state as can be seen in Figure 7.

The evidence base for ivermectin against COVID-19

To date, the efficacy of ivermectin in COVID-19 has been supported by the following:

1. Since 2012, multiple in vitro studies have demonstrated that Ivermectin inhibits the replication of many viruses, including influenza, *Zika, Dengue*, and others.[9–17]
2. Ivermectin inhibits SARS-CoV-2 replication and binding to host tissue through several observed and proposed mechanisms.[18]
3. Ivermectin has potent anti-inflammatory properties with in vitro data demonstrating profound

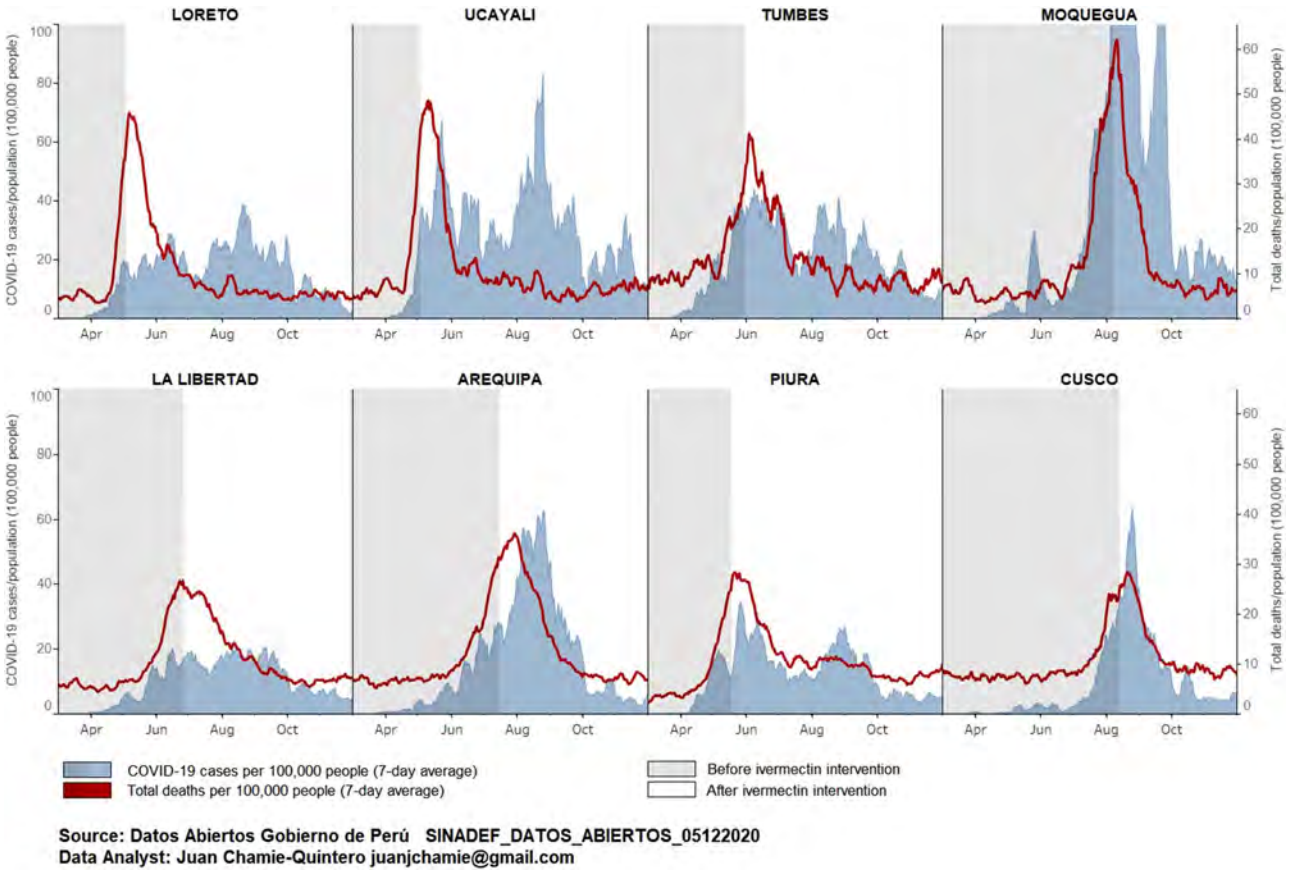

FIGURE 4. Decrease in total case incidences and total deaths/population of COVID-19 in the over 60 population among 8 Peruvian states after deploying mass ivermectin distribution campaigns.

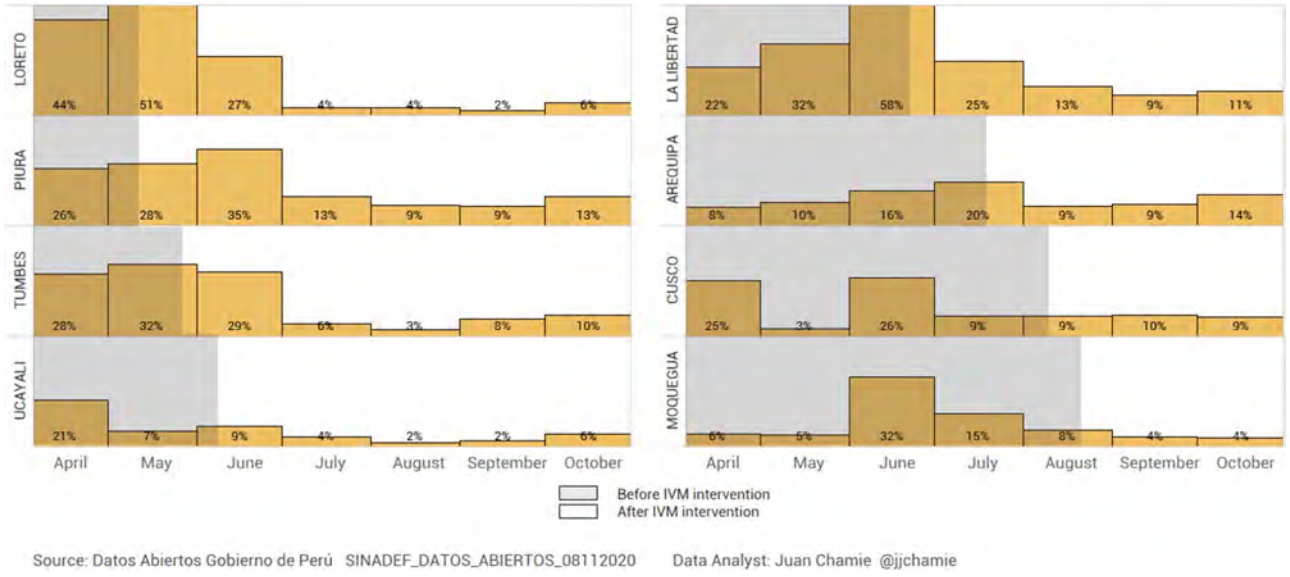

FIGURE 5. Daily total deaths, case fatalities, and case incidence for COVID-19 in populations of patients aged 60 and older for 8 states in Peru deploying early mass ivermectin treatments versus the state of Lima, including the capital city, where ivermectin treatment was applied months later.

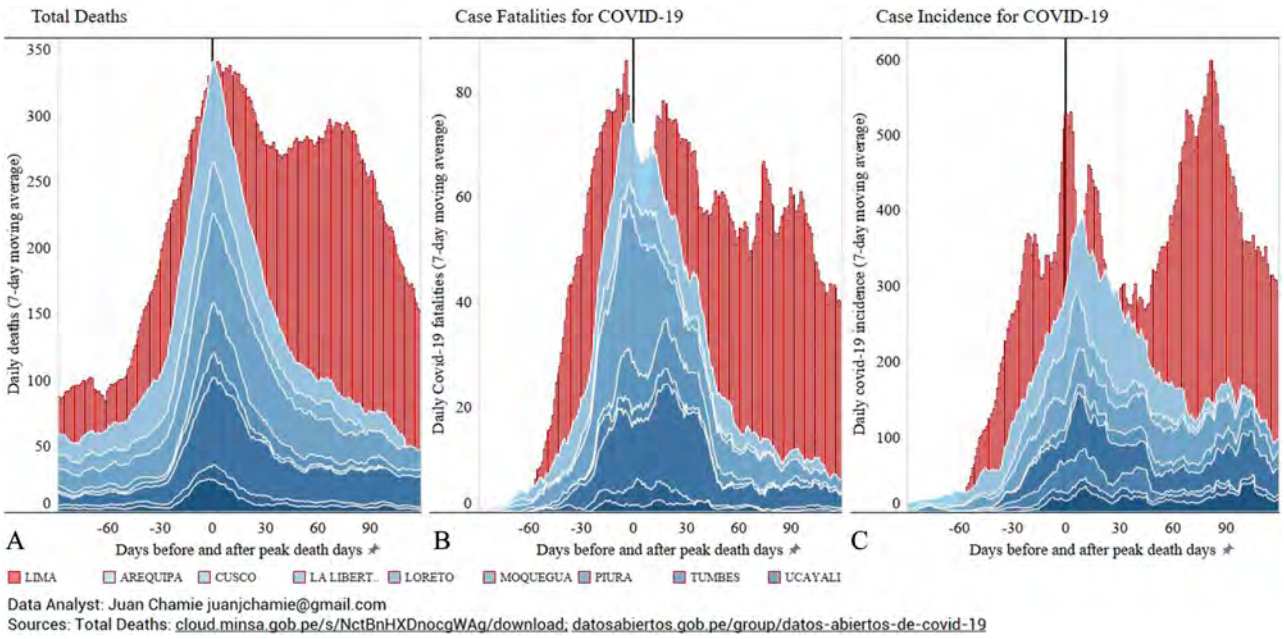

FIGURE 6. Covid-19 case fatalities and total deaths with and without mass ivermectin in different states of Peru.

inhibition of both cytokine production and transcription of nuclear factor-κB (NF-κB), the most potent mediator of inflammation.[37–39]

4. Ivermectin significantly diminishes viral load and protects against organ damage in multiple animal models when infected with SARS-CoV-2 or similar coronaviruses.[31,32]
5. Ivermectin prevents transmission and development of COVID-19 disease in those exposed to infected patients.[40–45]

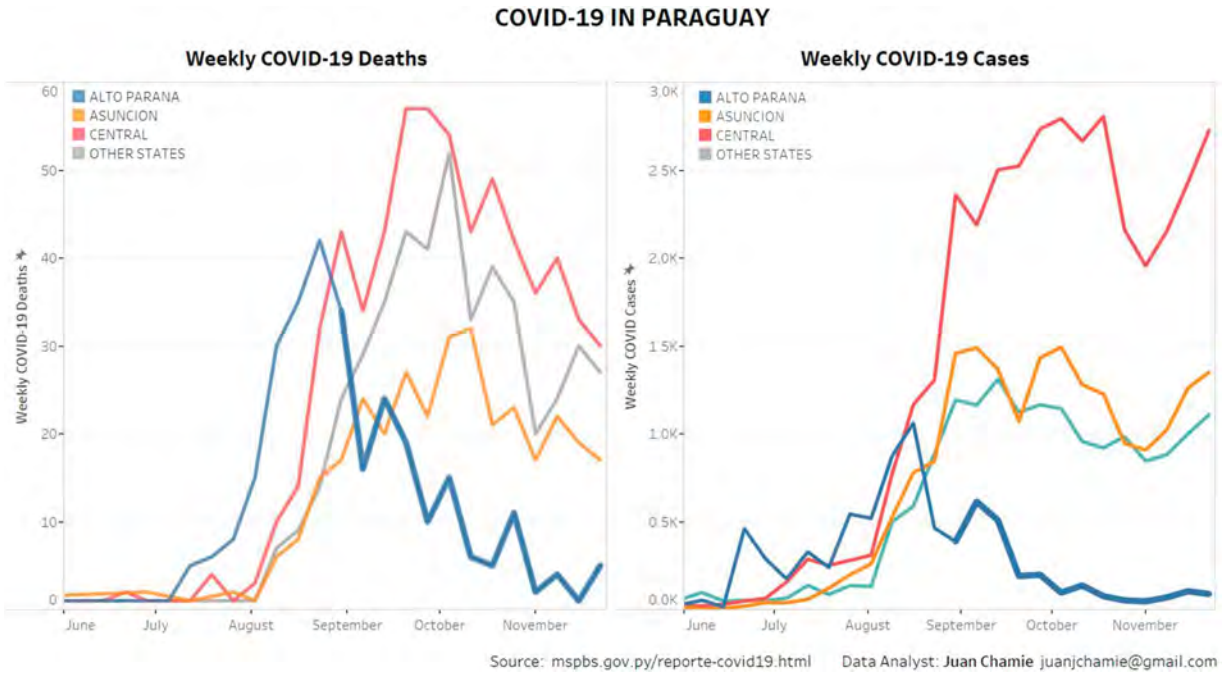

FIGURE 7. Paraguay – COVID-19 case counts and deaths in Alto Parana (bolded blue line) after ivermectin distribution began compared to other regions.

6. Ivermectin hastens recovery and prevents deterioration in patients with mild to moderate disease treated early after symptoms.[45,49–52,61,62]
7. Ivermectin hastens recovery and avoidance of ICU admission and death in hospitalized patients.[45,51,53,63–66]
8. Ivermectin reduces mortality in critically ill patients with COVID-19.[45,53,63]
9. Ivermectin leads to temporally associated reductions in case fatality rates in regions after ivermectin distribution campaigns.[48]
10. The safety, availability, and cost of ivermectin are nearly unparalleled given its low incidence of important drug interactions along with only mild and rare side effects observed in almost 40 years of use and billions of doses administered.[75]
11. The World Health Organization has long included ivermectin on its "List of Essential Medicines."

A summary of the statistically significant results from the above controlled trials are as follows:

Controlled trials in the prophylaxis of COVID-19 (8 studies)

1. All 8 available controlled trial results show statistically significant reductions in transmission.
2. Three RCTs with large statistically significant reductions in transmission rates, N = 774 patients.[44–46]
3. Five OCTs with large statistically significant reductions in transmission rates, N = 2052 patients.[40–43,47]

Controlled trials in the treatment of COVID-19 (19 studies)

1. Five RCTs with statistically significant impacts in time to recovery or hospital length of stay.[45,49,53,64,65]
2. One RCT with a near statistically significant decrease in time to recovery, $P = 0.07$, N = 130.[54]
3. One RCT with a large, statistically significant reduction in the rate of deterioration or hospitalization, N = 363.[49]
4. Two RCTs with a statistically significant decrease in viral load, days of anosmia, and cough, N = 85.[57,60]
5. Three RCTs with large, statistically significant reductions in mortality (N = 695).[45,60,65]
6. One RCT with a near statistically significant reduction in mortality, $P = 0.052$ (N = 140).[53]
7. Three OCTs with large, statistically significant reductions in mortality (N = 1688).[51,63,66]

Safety of ivermectin

Numerous studies report low rates of adverse events, with the majority mild, transient, and largely attributed to the body's inflammatory response to the death of the parasites and include itching, rash, swollen lymph nodes, joint paints, fever, and headache.[75] In a study that combined results from trials including more than 50,000 patients, serious events occurred in less than 1% and largely associated with administration in Loa loa.[76] Furthermore, according to the pharmaceutical reference standard *Lexicomp*, the only medications contraindicated for use with ivermectin are the concurrent administration of antituberculosis and cholera vaccines while the anticoagulant warfarin would require dose monitoring. Another special caution is that immunosuppressed or organ transplant patients who are on calcineurin inhibitors, such as tacrolimus or cyclosporine, or the immunosuppressant sirolimus should have close monitoring of drug levels when on ivermectin given that interactions exist that can affect these levels. A longer list of drug interactions can be found on the *drugs.com* database, with nearly all interactions leading to a possibility of either increased or decreased blood levels of ivermectin. Given studies showing tolerance and lack of adverse effects in human subjects given escalating high doses of ivermectin, toxicity is unlikely, although a reduced efficacy because of decreased levels may be a concern.[77]

Concerns of safety in the setting of liver disease are unfounded given that, to the best of our knowledge, only 2 cases of liver injury have ever been reported in association with ivermectin, with both cases rapidly resolved without need for treatment.[78,79] Furthermore, no dose adjustments are required in patients with liver disease. Some have described ivermectin as potentially neurotoxic, yet one study performed a search of a global pharmaceutical database and found only 28 cases among almost 4 billion doses with serious neurological adverse events, such as ataxia, altered consciousness, seizure, or tremor.[80] Potential explanations included the effects of concomitantly administered drugs that increase absorption past the blood–brain barrier or polymorphisms in the mdr-1 gene. However, the total number of reported cases suggests that such events are exceedingly rare. Finally, ivermectin has been used safely in pregnant women, children, and infants.

DISCUSSION

Currently, as of December 14, 2020, there is accumulating evidence that demonstrates both the safety and efficacy of ivermectin in the prevention and treatment of COVID-19. Large-scale epidemiologic analyses validate the findings of in vitro, animal, prophylaxis, and clinical studies. Epidemiologic data from regions of the world with widespread ivermectin use have demonstrated a temporally associated reduction in case counts, hospitalizations, and fatality rates.

In the context of ivermectin's long-standing safety record, low cost, and wide availability along with the

consistent, reproducible, large magnitude of findings on transmission rates, need for hospitalization, and mortality, widespread deployment in both prevention and treatment has been proposed. Although a subset of trials are of an observational design, it must be recognized that in the case of ivermectin (1) half of the trials used a randomized controlled trial design (12 of the 24 reviewed above) and (2) observational and randomized trial designs reach equivalent conclusions on average as reported in a large Cochrane review of the topic from 2014.[81] In particular, OCTs that use propensity-matching techniques (as in the Rajter study from Florida) find near identical conclusions to later-conducted RCTs in many different disease states, including coronary syndromes, critical illness, and surgery.[82–84] Similarly, as evidenced in the prophylaxis (Figure 1) and treatment trial (Figures 2 and 3) meta-analyses as well as the summary trials table (Table 3), the entirety of the benefits found in both OCT and RCT trial designs aligns in both direction and magnitude of benefit. Such a consistency of benefit among numerous trials of varying sizes designs from multiple different countries and centers around the world is unique and provides strong, additional support.

The continued challenges faced by health care providers in deciding on appropriate therapeutic interventions in patients with COVID-19 would be greatly eased if more updated and commensurate evidence-based guidance came from the leading governmental health care agencies. Currently, in the United States, the treatment guidelines for COVID-19 are issued by the National Institutes of Health. Their most recent recommendation on the use of ivermectin in patients with COVID-19 was last updated on February 11, 2021, where they found that "there was insufficient evidence to recommend for or against ivermectin in COVID-19." For a more definitive recommendation to be issued by major leading public health agencies (PHA), it is apparent that even more data on both the quality and quantity of trials are needed, even during a global health care emergency, and in consideration of a safe, oral, low-cost, widely available and deployable intervention such as ivermectin.

Fortunately, large teams sponsored by 2 different organizations have embarked on this effort. One team, sponsored by the Unitaid/WHO's ACT Accelerator Program and led by the University of Liverpool Senior Research Fellow Dr. Andrew Hill, is performing a systematic review and meta-analysis focused solely on ivermectin treatment RCTs in COVID-19. Although a preliminary meta-analysis of 17 RCTs was posted to a preprint server in February, it is expected that by March 19, 2021, results from approximately 27–29 RCTs including almost 4500 patients will be presented to the WHO Guidelines Committee and that the epidemiologic studies reviewed above by Chamie et al were already presented to the committee in early March (personal communication with Dr. Andrew Hill). It is important to note that on February 5, the WHO Guidelines Committee announced that they had begun a review of the accumulating ivermectin data and expected to arrive at their own formal treatment recommendation within 4–6 weeks. If the above benefits in clinical outcomes continue to be reported in the remaining trials, it is hoped that this almost doubling of the current supportive evidence base would merit a recommendation for use by the WHO, NIH, and other PHA's would be forthcoming.

Because of the urgency of the pandemic, and in response to the surprising persistent inaction by the leading PHA's, the British Ivermectin Recommendation Development Panel was recently coordinated by the Evidence-Based Medicine Consultancy Ltd to more rapidly formulate an ivermectin treatment guideline using the standard guideline development process followed by the WHO. Made up of long-time research consultants to numerous national and international public health organizations including the WHO, they convened both a steering committee and a technical working group that then performed a systematic review and meta-analysis. On February 12, 2021, a meeting was held that included an international consortium of 75 practitioners, researchers, specialists, and patient representatives representing 16 countries and most regions of the world. This Recommendation Development Panel was presented the results of the meta-analysis of 18 treatment RCTs and 3 prophylaxis RCTs including more than 2500 patients along with a summary of the observational trials and the epidemiologic analyses related to regional ivermectin use. After a discussion period, a vote was held on multiple aspects of the data on ivermectin, according to standard WHO guideline development processes. The Panel *found the certainty of evidence for ivermectin's effects on survival to be strong and they recommended unconditional adoption for use in the prophylaxis and treatment of COVID-19.*

In summary, based on the totality of the trials and epidemiologic evidence presented in this review along with the preliminary findings of the Unitaid/WHO meta-analysis of treatment RCTs and the guideline recommendation from the international BIRD conference, ivermectin should be globally and systematically deployed in the prevention and treatment of COVID-19.

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REVIEW

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Pathophysiological Basis and Rationale for Early Outpatient Treatment of SARS-CoV-2 (COVID-19) Infection

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ABSTRACT

Approximately 9 months of the severe acute respiratory syndrome coronavius-2 (SARS-CoV-2 [COVID-19]) spreading across the globe has led to widespread COVID-19 acute hospitalizations and death. The rapidity and highly communicable nature of the SARS-CoV-2 outbreak has hampered the design and execution of definitive randomized, controlled trials of therapy outside of the clinic or hospital. In the absence of clinical trial results, physicians must use what has been learned about the pathophysiology of SARS-CoV-2 infection in determining early outpatient treatment of the illness with the aim of preventing hospitalization or death. This article outlines key pathophysiological principles that relate to the patient with early infection treated at home. Therapeutic approaches based on these principles include 1) reduction of reinoculation, 2) combination antiviral therapy, 3) immunomodulation, 4) antiplatelet/antithrombotic therapy, and 5) administration of oxygen, monitoring, and telemedicine. Future randomized trials testing the principles and agents discussed will undoubtedly refine and clarify their individual roles; however, we emphasize the immediate need for management guidance in the setting of widespread hospital resource consumption, morbidity, and mortality.

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KEYWORDS: Ambulatory treatment; Anticoagulant; Anti-inflammatory; Antiviral; COVID-19; Critical care; Epidemiology; Hospitalization; Mortality; SARS-CoV-2

The pandemic of severe acute respiratory syndrome coronavius-2 (SARS-CoV-2 [COVID-19]) is rapidly expanding across the world with each country and region developing distinct epidemiologic patterns in terms of frequency, hospitalization, and death. There has been considerable focus on 2 major areas of response to the pandemic: containment of the spread of infection and reducing inpatient mortality.

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These efforts, although well-justified, have not addressed the ambulatory patient with COVID-19 who is at risk for hospitalization and death. The current epidemiology of rising COVID-19 hospitalizations serves as a strong impetus for an attempt at treatment in the days or weeks before a hospitalization occurs.[1] Most patients who arrive to the hospital by emergency medical services with COVID-19 do not initially require forms of advanced medical care.[2] Once hospitalized, approximately 25% require mechanical ventilation, advanced circulatory support, or renal replacement therapy. Hence, it is conceivable that some, if not a majority, of hospitalizations could be avoided with a treat-at-home first approach with appropriate telemedicine monitoring and access to oxygen and therapeutics.[3]

As in all areas of medicine, the large randomized, placebo-controlled, parallel group clinical trial in appropriate patients at risk with meaningful outcomes is the theoretical gold standard for recommending therapy. These standards are not sufficiently rapid or responsive to the COVID-19 pandemic.[4] One could argue the results of definitive trials were needed at the outset of the pandemic, and certainly are needed now with more than 1 million cases and 500,000 deaths worldwide.[5] Because COVID-19 is highly communicable, many ambulatory clinics do not care for patients in face-to-face visits, and these patients are commonly declined by pharmacies, laboratories, and imaging centers. On May 14, 2020, after about 1 million cases and 90,000 deaths in the United States had already occurred, the National Institutes of Health (NIH) announced it was launching an outpatient trial of hydroxychloroquine (HCQ) and azithromycin in the treatment of COVID-19.[6] A month later, the agency announced it was closing the trial because of the lack of enrollment with only 20 of 2000 patients recruited.[7] No safety concerns were associated with the trial. This effort serves as the best current working example of the lack of feasibility of outpatient trials for COVID-19. It is also a strong signal that future ambulatory trial results are not imminent or likely to report soon enough to have a significant public health impact on clinical outcomes.[8]

If clinical trials are not feasible or will not deliver timely guidance to clinicians or patients, then other scientific information bearing on medication efficacy and safety needs to be examined. Cited in this article are more than a dozen studies of various designs that have examined a range of existing medications. Thus, in the context of present knowledge, given the severity of the outcomes and the relative availability, cost, and toxicity of the therapy, each physician and patient must make a choice: watchful waiting in self-quarantine or empiric treatment with the aim of reducing hospitalization and death. Because COVID-19 expresses a wide spectrum of illness progressing from asymptomatic to symptomatic infection to fulminant adult respiratory distress syndrome and multiorgan system failure, there is a need to individualize therapy according to what has been learned about the pathophysiology of human SARS-CoV-2 infection.[9] It is beyond the scope of this article to review every preclinical and retrospective study of proposed COVID-19 therapy. Hence, the agents proposed are those that have appreciable clinical support and are feasible for administration in the ambulatory setting. SARS-CoV-2 as with many infections may be amenable to therapy early in its course but is probably not responsive to the same treatments very late in the hospitalized and terminal stages of illness.[10]

For the ambulatory patient with recognized early signs and symptoms of COVID-19, often with nasal real-time reverse transcription or oral antigen testing pending, the following 4 principles could be deployed in a layered and escalating manner depending on clinical manifestations of COVID-19-like illness[11] and confirmed infection: 1) reduction of reinoculation, 2) combination antiviral therapy, 3) immunomodulation, and 4) antiplatelet/antithrombotic therapy. Because the results of testing could take up to a week to return, treatment can be started before the results are known. For patients with cardinal features of the syndrome (ie, fever, body aches, nasal congestion, loss of taste and smell, etc.) and suspected false-negative testing, treatment can be the same as those with confirmed COVID-19.[11] Future randomized trials are expected to confirm, reject, refine, and expand these principles. In this article, they are set forth in emergency response to the growing pandemic as shown in Figure 1.

CLINICAL SIGNIFICANCE

- COVID-19 hospitalizations and death can be reduced with outpatient treatment.
- Principles of COVID-19 outpatient care include: 1) reduction of reinoculation, 2) combination antiviral therapy, 3) immunomodulation, 4) antiplatelet/antithrombotic therapy 5) administration of oxygen, monitoring, and telemedicine.
- Future randomized trials will undoubtedly refine and clarify ambulatory treatment, however we emphasize the immediate need for management guidance in the current crisis of widespread hospital resource consumption, morbidity, and mortality.

CONTROL OF CONTAGION

A major goal of self-quarantine is the control of contagion.[12] Many sources of information suggest the main place of viral transmission occurs in the home.[13] Facial covering for all contacts within the home as well as frequent use of hand sanitizer and hand washing is mandatory. Sterilizing surfaces such as countertops, door handles, phones, and other devices is advised. When possible, other close contacts can move out of the domicile and temporarily stay with others not ill with SARS-CoV-2. Findings from

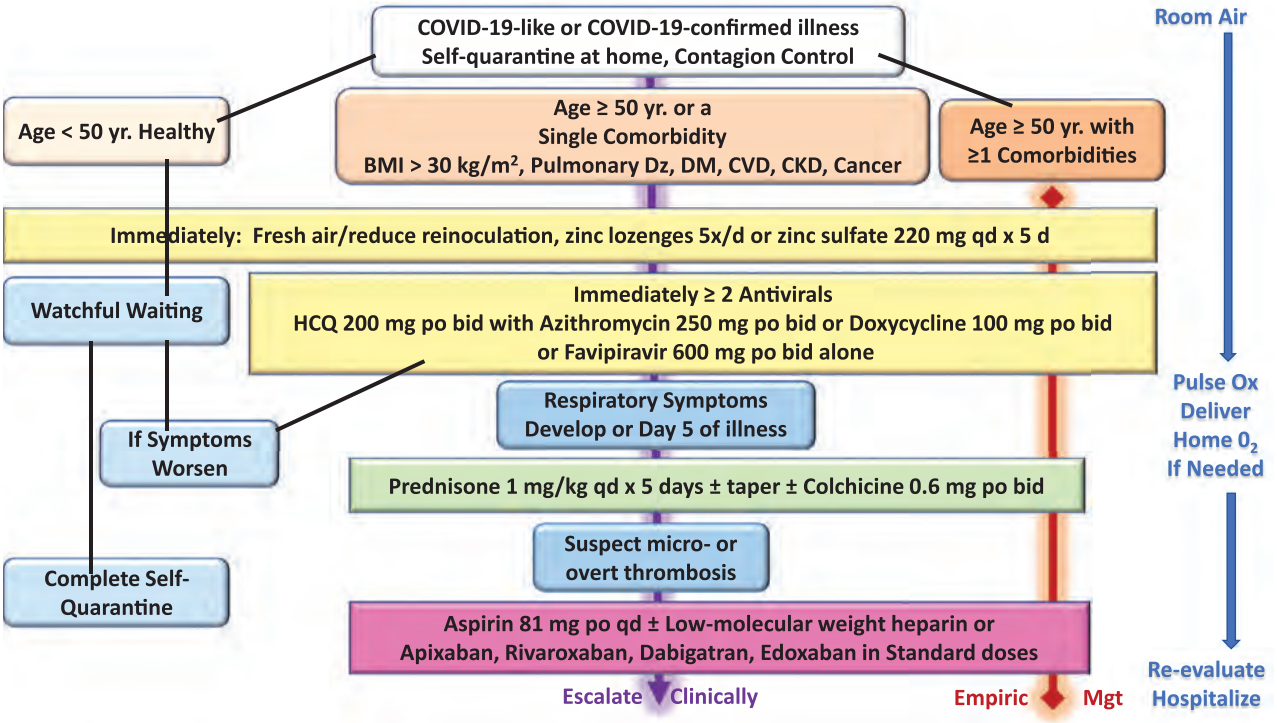

Figure 1 Treatment algorithm for COVID-19-like and confirmed COVID-19 illness in ambulatory patients at home in self-quarantine. BMI = body mass index; CKD = chronic kidney disease; CVD = cardiovascular disease; DM = diabetes mellitus; Dz = disease; HCQ = hydroxychloroquine; Mgt = management; O_2 = oxygen; Ox = oximetry; Yr = year.

multiple studies indicate that policies concerning control of the spread of SARS-CoV-2 are effective and extension into the home as the most frequent site of viral transfer is paramount.[14]

REDUCTION OF SELF-REINOCULATION

It is well-recognized that COVID-19 exists outside the human body in a bioaerosol of airborne particles and droplets. Because exhaled air in an infected person is considered to be "loaded" with inoculum, each exhalation and inhalation is effectively reinoculation.[15] In patients who are hospitalized, negative pressure is applied to the room air largely to reduce spread outside of the room. We propose that fresh air could reduce reinoculation and potentially reduce the severity of illness and possibly reduce household spread during quarantine. This calls for open windows, fans for aeration, or spending long periods of time outdoors away from others with no face covering to disperse and not reinhale the viral bioaerosol.

COMBINATION ANTIVIRAL THERAPY

Rapid and amplified viral replication is the hallmark of most acute viral infections. By reducing the rate, quantity, or duration of viral replication, the degree of direct viral injury to the respiratory epithelium, vasculature, and organs may be lessened.[16] Additionally, secondary processes that depend on viral stimulation, including the activation of inflammatory cells, cytokines, and coagulation, could potentially be lessened if viral replication is attenuated. Because no form of readily available medication has been designed specifically to inhibit SARS-CoV-2 replication, 2 or more of the nonspecific agents listed here can be entertained. None of the approaches listed have specific regulatory approved advertising labels for their manufacturers; thus all would be appropriately considered acceptable "off-label" use.[17]

Zinc Lozenges and Zinc Sulfate

Zinc is a known inhibitor of coronavirus replication. Clinical trials of zinc lozenges in the common cold have demonstrated modest reductions in the duration and or severity of symptoms.[18] By extension, this readily available nontoxic therapy could be deployed at the first signs of COVID-19.[19] Zinc lozenges can be administered 5 times a day for up to 5 days and extended if needed if symptoms persist. The amount of elemental zinc lozenges is <25% of that in a single 220-mg zinc sulfate daily tablet. This dose of zinc sulfate has been effectively used in combination with antimalarials in early treatment of high-risk outpatients with COVID-19.[20]

Antimalarials

Hydroxychloroquine (HCQ) is an antimalarial/anti-inflammatory drug that impairs endosomal transfer of virions within human cells. HCQ is also a zinc ionophore that

conveys zinc intracellularly to block the SARS-CoV-2 RNA-dependent RNA polymerase, which is the core enzyme of the virus replication.[21] The currently completed retrospective studies and randomized trials have generally shown these findings: 1) when started late in the hospital course and for short durations of time, antimalarials appear to be ineffective, 2) when started earlier in the hospital course, for progressively longer durations and in outpatients, antimalarials may reduce the progression of disease, prevent hospitalization, and are associated with reduced mortality.[22–25] In a retrospective inpatient study of 2541 patients hospitalized with COVID-19, therapy associated with an adjusted reduction in mortality was HCQ alone (hazard ratio [HR] = 0.34, 95% confidence interval [CI] 0.25-0.46, P <0.001) and HCQ with azithromycin (HR = 0.29, 95% CI 0.22-0.40, P <0.001).[23] HCQ was approved by the US Food and Drug Administration in 1955, has been used by hundreds of millions of people worldwide since then, is sold over the counter in many countries, and has a well-characterized safety profile that should not raise undue alarm.[25,26] Although asymptomatic QT prolongation is a well-recognized and infrequent (<1%) complication of HCQ, it is possible that in the setting of acute illness symptomatic arrhythmias could develop. Data safety and monitoring boards have not declared safety concerns in any clinical trial published to date. Rare patients with a personal or family history of prolonged QT syndrome and those on additional QT prolonging, contraindicated drugs (eg, dofetilide, sotalol) should be treated with caution and a plan to monitor the QTc in the ambulatory setting. A typical HCQ regimen is 200 mg bid for 5 days and extended to 30 days for continued symptoms. A minimal sufficient dose of HCQ should be used, because in excessive doses the drug can interfere with early immune response to the virus.

Azithromycin

Azithromycin is a commonly used macrolide antibiotic that has antiviral properties mainly attributed to reduced endosomal transfer of virions as well as established anti-inflammatory effects.[27] It has been commonly used in COVID-19 studies initially based on French reports demonstrating markedly reduced durations of viral shedding, fewer hospitalizations, and reduced mortality combination with HCQ as compared to those untreated.[28,29] In the large inpatient study (n = 2451) discussed previously, those who received azithromycin alone had an adjusted HR for mortality of 1.05, 95% CI 0.68-1.62, and P = 0.83.[23] The combination of HCQ and azithromycin has been used as standard of care in other contexts as a standard of care in more than 300,000 older adults with multiple comorbidities.[30] This agent is well-tolerated and like HCQ can prolong the QTc in <1% of patients. The same safety precautions for HCQ listed previously could be extended to azithromycin with or without HCQ. Azithromycin provides additional coverage of bacterial upper respiratory pathogens that could potentially play a role in concurrent or secondary infection. Thus, this agent can serve as a safety net for patients with COVID-19 against clinical failure of the bacterial component of community-acquired pneumonia.[31,32] The same safety precautions for HCQ could be extended to azithromycin with or without HCQ. Because both HCQ and azithromycin have small but potentially additive risks of QTc prolongation, patients with known or suspected arrhythmias or taking contraindicated medications or should have more thorough workup (eg, review of baseline electrocardiogram, imaging studies, etc.) before receiving these 2 together. One of many dosing schemes is 250 mg po bid for 5 days and may extend to 30 days for persistent symptoms or evidence of bacterial superinfection.

Doxycycline

Doxycycline is another common antibiotic with multiple intracellular effects that may reduce viral replication, cellular damage, and expression of inflammatory factors.[33,34] This drug has no effect on cardiac conduction and has the main caveat of gastrointestinal upset and esophagitis. As with azithromycin, doxycycline has the advantage of offering antibacterial coverage for superimposed bacterial infection in the upper respiratory tract. Doxycycline has a high degree of activity against many common respiratory pathogens including *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis*, anaerobes such as Bacteroides and anaerobic/microaerophilic streptococci and atypical agents like Legionella, *Mycoplasma pneumoniae*, and *Chlamydia pneumoniae*.[34] One of many dosing schemes is 200 mg po followed by 100 mg po bid for 5 days and may extend to 30 days for persistent symptoms or evidence of bacterial superinfection. Doxycycline may be useful with HCQ for patients in whom the HCQ-azithromycin combination is not desired.

Favipiravir

Favipiravir, an oral selective inhibitor of RNA-dependent RNA polymerase, is approved for ambulatory use in COVID-19 in Russia, India, and other countries outside of the United States.[35] It has been previously used for treatment of some life-threatening infections such as Ebola virus, Lassa virus, and rabies. Its therapeutic efficacy has been proven in these diseases.[36] Like, the antimalarials and antibiotics, favipiravir has no large-scale randomized trials completed at this time, given the short time frame of the pandemic. A dose administration could be 1600 mg po bid on day 1, following by 600 mg po bid for 14 days.[37]

IMMUNOMODULATORS

The manifestations of COVID-19 that prompt hospitalization and that may well lead to multiorgan system failure are attributed to a cytokine storm. The characteristic profile of a patient acutely ill with COVID-19 includes leukocytosis with a relative neutropenia. These patients have higher

serum level of cytokines (ie, TNF-α, IFN-γ, IL-1β, IL-2, IL-4, IL-6, and IL-10) and C-reactive protein than control individuals. Among patients with COVID-19, serum IL-6 and IL-10 levels appear even more elevated in the critically ill.[38] As with any acute inflammatory state, early treatment with immunomodulators is expected to impart greater benefit. In COVID-19, some of the first respiratory findings are nasal congestion, cough, and wheezing. These features are due to excess inflammation and cytokine activation. Early use of corticosteroids is a rational intervention for patients with COVID-19 with these features as they would be in acute asthma or reactive airways disease.[39,40] The RECOVERY trial randomized 6425 hospitalized patients with COVID-19 in a 2:1 ratio to dexamethasone 6 mg po/IV daily for up to 10 days and found dexamethasone reduced mortality (HR = 0.65, 95% CI 0.51-0.82, P <0.001).[41] One potential dosing scheme for outpatients starting on day 5 or the onset of respiratory symptoms is prednisone 1 mg/kg given daily for 5 days with or without a subsequent taper.

Colchicine

Colchicine is a nonsteroidal antimitotic drug that blocks metaphase by binding to the ends of microtubules to prevent the elongation of the microtubule polymer. This agent has proven useful in gout and idiopathic recurrent pericarditis. The GRECCO-19 randomized open-label trial in 105 hospitalized patients with COVID-19 found that colchicine was associated with a reduction in D-dimer levels and improved clinical outcomes.[42] The clinical primary end point (2-point change in World Health Organization ordinal scale) occurred in 14.0% in the control group (7 of 50 patients) and 1.8% in the colchicine group (1 of 55 patients) (odds ratio, 0.11; 95% CI, 0.01-0.96; P = 0.02).[43] Because the short-term safety profile is well understood, it is reasonable to consider this agent along with corticosteroids in an attempt to reduce the effects of cytokine storm. A dosing scheme of 1.2 mg po, followed by 0.6 mg po bid for 3 weeks can be considered.

ANTIPLATELET AGENTS AND ANTITHROMBOTICS

Multiple studies have described increased rates of pathological macro- and micro-thrombosis.[44,45] Patients with COVID-19 have described chest heaviness associated with desaturation that suggests the possibility of pulmonary thrombosis.[46] Multiple reports have described elevated D-dimer levels in acutely ill patients with COVID-19, which has been consistently associated with increased risk of deep venous thrombosis and pulmonary embolism.[47–49] Necropsy studies have described pulmonary microthrombosis in COVID-19.[50] These observations support the notion that endothelial injury and thrombosis play a role oxygen desaturation, a cardinal reason for hospitalization and supportive care.[47] Based on this pathophysiologic rationale, aspirin 81 mg daily can be administered as an initial antiplatelet and anti-inflammatory agent.[51,52] Ambulatory patients can be additionally treated with subcutaneous low-molecular-weight heparin or with short-acting novel anticoagulant drugs in dosing schemes similar to those use in outpatient thromboprophylaxis. In a retrospective study of 2773 inpatients with COVID-19, 28% received anticoagulant therapy within 2 days of admission, and despite being used in more severe cases, anticoagulant administration was associated with a reduction in mortality (HR = 0.86 per day of therapy, 95% CI: 0.82-0.89; P <0.001). Additional supportive data on the use anticoagulants reducing mortality has been reported in hospitalized patients with elevated D-dimer levels and higher comorbidity scores.[53] Many acutely ill outpatients also have general indications for venous thromboembolism prophylaxis applicable to COVID-19.[54]

DELIVERY OF OXYGEN AND MONITORING

Because ambulatory centers and clinics have been reticent to have face-to-face visits with patients with COVID-19, telemedicine is a reasonable platform for monitoring. Clinical impressions can be gained with audio and video interviews by the physician with the patient. Supplemental information, including vital signs and symptoms, will be important to guide the physician. A significant component of safe outpatient management is maintenance of arterial oxygen saturation on room air or prescribed home oxygen under direct supervision by daily telemedicine with escalation to hospitalization for assisted ventilation if needed. Self-proning could be entertained for confident patients with good at-home monitoring.[55]

Many of the measures discussed in this article could be extended to seniors in COVID-19 treatment units in nursing homes and other nonhospital settings. This would leave the purposes of hospitalization to the administration of intravenous fluid and parenteral medication, assisted pressure or mechanical ventilation, and advanced mechanical circulatory support.

SUMMARY

Acute COVID-19 has a great range of clinical severity from asymptomatic to fatal. In the absence of clinical trials and guidelines, with hospitalizations and mortality mounting, it is prudent to deploy treatment for COVID-19 based on pathophysiological principles. We have proposed an algorithm based on age and comorbidities that allows for a large proportion to be monitored and treated at home during self-isolation with the aim of reducing the risks of hospitalization and death.

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FACT SHEET: COVID EXPERIMENTAL VACCINES 05/31/21

- ***The Vaccine Adverse Events Reports System (VAERS) Center for Disease Control (CDC) COVID Reports:*** **227,805 Reports Though May 14, 2021**
  Reported since the experimental vaccines for COVID 19
  - DEATHS: 4,201 - HOSPITALIZATIONS 12,625
  - URGENT CARE VISITS 29,707 - PRIMARY CARE VISITS 39,153
  - ANAPHYLACTIC REACTION 1,070 - BELLS PALSY 1,273
- *COVID 19 Survival Rates by Age Group (the percentage that will survive an infection) reported by CDC*
  0-19: 99.997%
  20-49 99.98%
  50-69: 99.5%
  70+: 94.6
- The Israeli Health Ministry reported that those above the age of 65 who receive the mRNA experimental Pfizer vaccine have an estimated 40x higher chance of dying due to the vaccine when compared to the chances of dying of COVID 19. For those below 65, the chances of dying from the vaccine are 260x higher than the chances of dying from COVID 19 (Delaney, 2021).
- It is now known that **the "active part" of the SAR-CoV2 virus is the spike protein** that binds to the ACE- 2 receptors of a cell causing damage to the endothelium (blood vessels) and impairs the function of the ACE – 2 enzyme that is important for cardiovascular health (Lei et al., 2020) (Kuba et al., 2005) (Zhang et al., 2021) Since **the spike protein (S protein**) is what the experimental vaccines have the recipients' cell manufacture (through synthetic RNA), or in the case of the Johnson and Johnson, carried via another virus, the safety of these experimental vaccines must be seriously questioned. Dr. Peter McCullough MPH MD professor of medicine at Texas A&M University (Baylor campus) the doctor with the most citations in the National Library of Medicine, and a leader on the topic of COVID 19**, has questioned the safety of the vaccines and suppression of proven treatments. He is warning not to proceed with the mass vaccination of children with these experimental vaccines** (McCullough, 2021).

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ADDENDUM MATERIAL
DATE 8/10/21 ITEM NO. A004

Jessica Kopfmann

From: Joanna Vercel [redacted]>
Sent: Wednesday, August 11, 2021 10:41 AM
To: COB_mail
Subject: Urgent: STOP THE MANDATE

To the tyrants on the board,
How dare you think that you all know better than the rest of us. You have NO right to tell me or any other healthcare professionals what is best to put in their body. Matt Heinz you are a disgrace to the medical profession. Have you forgotten that you took an oath to do no harm? You are blatantly ignoring the science and mounds of evidence from real physicians. I can't tell if you're just plain old ignorant or if you're down right evil. But it stops here! You nor any other board will ever tell me or anyone else what they must put in their bodies. There are plenty of resources that do not harm or have the side effects of the injection being offered. Instead of being a tyrant why don't you try actually being a doctor and treating your patients according to the FLCCC protocol. They've actually success with their patients. If you want to address something address the outrageous amounts of processed food peoples consume and their lack of vitamin D and C. Encourage people to eat pasture raised food from regenerative farmers. These things that profoundly effect ones immune system. Should you all be ignorant enough to pass such a stupid thing, know that it will not be upheld and you will be personally held accountable.

Joanna

Jessica Kopfmann

From: Ginny Hewitt [redacted]>
Sent: Wednesday, August 11, 2021 11:17 AM
To: COB_mail
Subject: STOP THE MANDATES!

Greetings,

As a registered nurse of 26 years currently licensed in the great state of Arizona, I am petitioning to stop mandatory covid jabs for health care professionals. As you are well aware, covid "vaccines" are currently approved under emergency authorization only and not FDA licensed.

To require a healthcare worker or anyone to be injected with a experimental "vaccine" is not only unconstitutional and illegal, it is medically reckless.

Thank you for in advance for your action to protect the health and personal rights of healthcare workers and all workers. Please feel free to contact me with questions at [redacted].

Respectfully,

Ginny Hewitt, MA, BSN, RN

Sent from my iPhone

Jessica Kopfmann

From: Ernesto Valencia <[redacted]>
Sent: Wednesday, August 11, 2021 11:25 AM
To: COB_mail
Subject: URGENT : STOP THE MANDATE !!!!!

Importance: High

AFTER WEIGHING ALL MY OPTIONS - AND CONSIDERING ALL - in the end - it is my choice - no mandates - no way !

This is clearly an evil agenda that will cripple our city financially and endanger all our lives by up ending the health care system and cause great harm to - all us citizens...

We love our nurses and health care workers that take care of us .

you claim to care about the citizens – then why are you trying to force them in to unsafe practices ...?

Let our trained health care professionals do what they love to do -

THERE ARE OTHER TRIED TESTED TRUE TREATMENTS - THAT WORK (been successful for decades) - why don't we talk about that ?

I suspect because they are inexpensive and readily available to anyone and they work ...so big Pharma cant benefit -

And it's a shame for any medical practitioner that knows this and is helping to suppress this information -

THEY MUST BE ON THE TAKE AS WELL (benefitting financially - makes you wonder where all the "incentive" money is coming from)

(instead of showing some appreciation to the frontline workers that worked thru the whole thing (unvaccinated)

OR ARE THEY UNDER SOME KIND OF PROFESSIONAL PRESSURE TO GO WITH THE FLOW ... BEING THREATENED WITH JOBS (maybe they don't know they are protected too)

Why not let them do what they have been doing - and without restrictions or discrimination or biased treatment -

these people - the health care workers - have been in the trenches the whole time - NO ONE GOT SICK !!!!

without vaccines (experimental shots of who knows what)

we all know the world is the laboratory - we don't want to be a guinea pig -

we have an army of local people that care about local people - let them treat (continue to treat) all our city with great care .

without mandates - (which are illegal) . – without restrictions (masking, social distancing) etcc.... We know this don't work -

this is absolute BS! And .....

THIS IS ABSOLUTELY ILLEGAL !!! WE CANNOT BE FORCED TO TAKE A SHOT WE DON'T WANT !

AS A CITIZEN OF THE UNITED STATES

I HAVE RIGHTS AND I AM PROTECTED BY THE CONSTITUTION OF THE UNITED STATES OF AMERICA

I GET TO CHOOSE WHAT I PUT INSIDE ME AND NO ONE ELSE -

INFORMED CONSENT - OR INFORMED DECISION - IT IS MY DECISION. MY DECISION ALONE

I HAVE A FEW REASONS HERE

These are just some reasons ....that in my mind.....and frankly I believe that any person with a little common sense ..... throw up a lot of red flags

with 99.98% survival rate hmmm I think a shot is Not necessary for what seems to be a typical virus

Unknown long term effects MRna Gene technology - (this is why we have trials and it takes a long time to study) ....NOT FAST TRACKED – look at all the side affects that are not being reported .... Gotta wonder why ???

UE - Listed as an ingredient - this was on the toxicology list of poisons.... Wow take this poison (wink wink just a little ...) NO THANK YOU !!!!

Fetal cell use goes against my Religious beliefs

Hidden list of unfamiliar ingredients... hmmmm wonder why ????

Criminal history of J&J , Phizer and Moderna – not someone I trust anyway ...

No history of vaccines from either producer – this is comical ... all of a sudden we make vaccines ..... NOT !!!!

Fast track to emergency use (after the products were already sold) documented proof of sales before bug even came to country ... wow No Shame !

Incentives (bribes) ..... really ??? – this would not be needed if the threat was real ... its all alie !!!

Shot does not prevent infection or transmission – so why bother ... and the lies of the DELTA VARIENT THERE IS NO SUCH TEST AVAILABLE ... MORE LIES - AND THEHIGHER #'S ARE FROM THE VACCINATED ....!!!

Adverse Events and deaths are unreported and un advertised / un disclosed - WHAT ELSE ARE THEY HIDING !!!!

Media censorship of Opposing experts – YOU HAVE TO ASK WHY ... WHATS THE COVER UP ... WHAT ABOUT INFORMED CONSENT !! WOW

Suppression of reports from Vaccine victims and families on social media ..... MOR BLACKBALLING / CENSORSHIP - THIS IS INSANE !

Censorship and bias is unprecedented - we see that big tech , big Pharma , the media , they are all in on it together ..... open your eyes -

THIS IS ILLEGAL AND CORRUPT AND I DO NOT WANT ANY PART OF IT - WE NEED TO SAVE OUR COUNTRY / OUR CONSTITUTION / OUR RIGHT S !!!!

No no no mandates !!!!

To the Called, the Chosen, and the Faithful.

MAY OUR FATHER IN HEAVEN –
BY HIS HOLY SPIRIT
RICHLY - BLESS YOU AND KEEP YOU –
IN JESUS – OUR GOD AND SAVIOR –

– see you at the wedding !

Ernesto Armando Valencia Jr.

Jessica Kopfmann

From: Paul LaValley [redacted]>
Sent: Wednesday, August 11, 2021 12:54 PM
To: Dist1@pima.gov; DIST2; District3; Dist4@pima.gov; Dist5@pima.gov; Chuck Huckelberry; COB_mail; Mable; Garret@790knst.com; KELLY@kellyjohnwalker.com
Subject: Re: Urgent! Stop The Mandates

Thank you for the kind replies. My final thoughts go out regarding the derogatory :Typhoid Mary" comments by the esteemed Doctor Matt "scrubs" Heinze,

I understand that the BOS will meet again early next week to re-vote on the topic of Mandates. I have two questions for Matt:
1) Will you once again become unhinged and call the decent folk, many of whom are constituents who happen to disagree with you, vulgar names again?
2) Will you either apologise or resign for embarrassing yourself, and insulting many thousands of decent folk in Pima County,, and millions nationwide?

Thanks again,
Paul.LaValley

On Wed, Aug 11, 2021 at 8:16 AM Paul LaValley [redacted]> wrote:
Dear Sirs,
I, like many, had the virus, thus I don't require the added protection of a vaccination with a 75% effective rate for a virus that has a 99.8% recovery rate...the recovery rate is higher than that if one uses the banned Therapeutics or Prophalactics' such as HCQ/Ivermectin.

It's LUNACY to even consider mandating this or any experimental drug with a growing history of adverse reactions, and death. especially since the borders are wide open...no mention of fixing that from you with a blanket travel ban. I don't care what loophole your lawyers may find in the law...it's wrong!

Why no mention of the deaths from Cigarette smoking, or traffic accidents? they kill more than the virus.

Do the right thing. Don't be a part of the biggest hoax ever put upon the American people.
Groups are forming as I speak. If you vote for this travesty of liberty, you will be protested, and exposed as the enemy of the people, which is what you will be if you vote for Mandatory Vaccinations aka: tyranny.

Sincerely,
Paul LaValley- Green Valley

Jessica Kopfmann

From: Julie G. [redacted]>
Sent: Wednesday, August 11, 2021 1:09 PM
To: COB_mail
Subject: URGENT: STOP THE VACCINE MANDATE

Jessica Kopfmann

From: Julie Grenier <grenier@email.arizona.edu>
Sent: Wednesday, August 11, 2021 1:10 PM
To: COB_mail
Subject: Urgent: Stop the Vaccine Mandate

Jessica Kopfmann

From: C Gotchey [redacted]>
Sent: Wednesday, August 11, 2021 1:19 PM
To: COB_mail
Subject: No mandatory vax!!!

Do your research! It's gene therapy it's not a vaccine! It does NOT give you immunity and it does NOT prevent you from spreading the virus! If you want to accept gene therapy for yourself that is your choice! You can not force medical experiments on people who
don't want it! If someone dies from the vaccine because you mandated it then make no mistake, you will be the fall guy! You are a government employee and you work for the people of Pima County. It is NOT your job to give out medical advice or to push medical experiments on human beings. Let the people choose. It is misinformation to tell people that the vaccine protects others. It does NOT! It also does NOT give you immunity! Maybe you ought to go to medical school before you start dishing out mandates about medicine that you clearly know nothing about!

Keep America free from you and your agenda!

Sent from my iPhone

Jessica Kopfmann

From: Michelle Sullivan [redacted]>
Sent: Wednesday, August 11, 2021 1:32 PM
To: COB_mail
Subject: Urgent - Stop The Mandates

These mandates are unconstitutional. We, the Citizens, do NOT consent. Stop overstepping what you were sent there to do. The Agenda is "We the People", not the UN WEF, ESG, or any other global directive.

Thank You,

Michelle Sullivan

Jessica Kopfmann

From: Heidi Mosier [redacted]>
Sent: Wednesday, August 11, 2021 1:50 PM
To: COB_mail
Subject: Covid mandate

I am emailing to make my voice heard and to share my disbelief that Pima county would even consider the Covid vaccine mandate.
The Healthcare workers were our heros on the front line and worked through this time without the vaccine. I am NOT against the vaccine for those who wish to get it BUT ABSOLUTELY believe it is wrong to make it mandatory for ANYONE who does not want to get It.

Please hear the voice of people and understand that Healthcare workers should NOT have to do this. Imagine the negative impact on the health system when you fire an already short staffed group. This choice would basically strain your ability to offer adequate care to those in Pima County. This will also impact an already weakened economic situation by adding to the unemployment rate.

Hear our voice!
Do the constitutional thing and allow Healthcare workers choose to be vaccinated on their own!

Heidi Mosier

Jessica Kopfmann

From: Marcia Grenier <[redacted]>
Sent: Wednesday, August 11, 2021 1:54 PM
To: COB_mail
Subject: stop the mandate

Matt Heinz is trying to make ALL healthcare workers get the covid shot by September 1!
Who the hell does he think he is to try to force people that do not want to put that shot in their bodies for whatever reason – religious, health, etc.
Where are our freedoms to decide what goes into our bodies? This is a democracy last I heard. It is scary place we're at to not have the freedom to choose.
We are willing to take the health risks by not putting these unwanted substances in our bodies.
We will have a severe shortage of healthcare workers in this county and this is more dangerous than Covid.
He is putting our whole county at rest reducing our healthcare workers. Does he care about that?
STOP THIS MADNESS!
Why doesn't he focus on the migrants that are infiltrating our city and county instead?
If He has his shot and wearing a mask which supposedly works – leave the rest of us alone!
Marcia Grenier
Pima County resident

Jessica Kopfmann

From: Peggy Rath [redacted]>
Sent: Wednesday, August 11, 2021 1:58 PM
To: COB_mail
Subject: Urgent: Stop the mandate

Please stop the mandate for healthcare workers to get the Moderna, Pfizer or J&J COVID vaccine.
It is unethical and immoral to mandate an emergency use vaccine or face termination. There must be voluntary consent, please.

Thank you,
Peggy Rath, PharmD

Sent from my iPhone

Jessica Kopfmann

From: BE Fuents [redacted]>
Sent: Wednesday, August 11, 2021 2:04 PM
To: COB_mail
Subject: Vaccine

Sent from my iPhone

Jessica Kopfmann

From: Michael Morris [redacted]>
Sent: Wednesday, August 11, 2021 2:10 PM
To: COB_mail
Subject: URGENT;STOP THE MANDATE

If we are not free to decide what goes into our bodies then what freedoms do we have? This madness needs to stop. I implore you to vote against any and all mandates.

Sent from my iPhone