



SARS-CoV-2 infects blood monocytes to activate NLRP3 and AIM2 inflammasomes, pyroptosis and cytokine release

Citation

Ingber, Jacob Jeffrey. 2021. SARS-CoV-2 infects blood monocytes to activate NLRP3 and AIM2 inflammasomes, pyroptosis and cytokine release. Master's thesis, Harvard Medical School.

Link

<https://nrs.harvard.edu/URN-3:HUL.INSTREPOS:37368635>

Terms of use

This article was downloaded from Harvard University's DASH repository, and is made available under the terms and conditions applicable to Other Posted Material (LAA), as set forth at

<https://harvardwiki.atlassian.net/wiki/external/NGY5NDE4ZjgzNTc5NDQzMGIzZWZhMGFIOWI2M2EwYTg>

Accessibility

<https://accessibility.huit.harvard.edu/digital-accessibility-policy>

Share Your Story

The Harvard community has made this article openly available.
Please share how this access benefits you. [Submit a story](#)

**SARS-CoV-2 infects blood monocytes to activate NLRP3 and AIM2 inflammasomes,
pyroptosis and cytokine release**

Jacob Jeffrey Ingber

A Thesis Submitted to the Faculty of

The Harvard Medical School

in Partial Fulfillment of the Requirements

for the Degree of Master of Medical Sciences in Immunology

Harvard University

Boston, Massachusetts

May, 2021

**SARS-CoV-2 infects blood monocytes to activate NLRP3 and AIM2 inflammasomes,
pyroptosis and cytokine release**

Abstract

The COVID-19 pandemic caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has infected over 100 million people and caused more than 2 million deaths worldwide. Although most infected people develop mild or moderate disease, a subset of mostly older patients develop severe life-threatening disease and die. What causes severe disease and inflammation is not well understood. Severe COVID-19 is accompanied by uncontrolled inflammation with cytokine storm resembling what occurs during sepsis. The severe inflammation observed during sepsis is the result of inflammatory cell death – pyroptosis – that releases inflammatory mediators, including interleukin-1 β (IL-1 β), interleukin-18 (IL-18) and cellular alarmins.

Monocytes are sentinel blood cells that alert the immune system to invasive infection and danger. Pyroptosis and the release of inflammatory IL-1 family cytokines relies on inflammasome activation of caspase-1 and gasdermin D (GSDMD), which forms pores in cell membranes. Here, my co-workers and I show that approximately 10% of circulating monocytes from COVID-19 patients are infected with SARS-CoV-2 and undergo pyroptosis. Signs of pyroptosis including IL-1 family cytokines and LDH in the plasma of COVID-19 patients correlate with disease severity. Both *in vivo* and *ex vivo* monocyte infection activates the NLRP3 and AIM2 inflammasomes, caspase-1, and GSDMD cleavage and re-localization to the cell

membrane. Importantly, *ex vivo* infection of healthy donor monocytes is enhanced by anti-SARS-CoV-2 antibodies or patient plasma.

Taken together, these results implicate antibody-dependent uptake of viral particles in monocyte infection, leading to inflammatory cytokine release and pyroptosis, which may be a major contributor to the cytokine release syndrome (CRS), or “cytokine storm,” seen in severe COVID-19 patients. These findings also suggest that blocking cytokine release and pyroptosis may prevent cytokine storm and severe disease.

Table of Contents

Abstract	ii
Table of Contents	iv
List of Figures	vi
Acknowledgements	vii
Chapter 1: Background	
1.1 Introduction	1
1.2 The COVID-19 Pandemic	2
1.3 Previous SARS-like coronaviruses	6
1.4 The SARS-CoV-2 Genome	8
1.5 The Anti-Viral Response	9
1.6 Inflammatory Cell Death	11
1.7 Conclusions	14
Chapter 2: Results	
2.1 Introduction	16
2.2 Materials and Methods	16
2.3 Results	27
2.4 Conclusion	42
2.5 Supplemental Data	44
Chapter 3: Discussion	
3.1 Overview	47
3.2 Significance of Findings	48

3.3 Remaining Questions	48
3.4 Conclusions	55
Bibliography	56

Figures

Figure 1. Clinical presentation of severe COVID-19.	4
Figure 2. Genome organization of 5 human <i>Betacoronaviruses</i> .	7
Figure 3. Overview of the major types of immune responses.	10
Figure 4. Overview of NLRP3 inflammasome activation.	14
Figure 5. Circulating monocytes and plasma of COVID-19 patients show signs of pyroptosis.	29
Figure 6. Plasma markers of pyroptosis correlate with severe COVID-19.	31
Figure 7. Circulating Monocytes have activated NLRP3 and AIM2 inflammasomes.	35
Figure 8. COVID-19 patient monocytes express ACE2 and CD147.	37
Figure 9. Circulating monocytes are infected with SARS-CoV-2 and undergo pyroptosis.	38
Figure 10. <i>In vitro</i> infection of healthy donor monocytes.	41
Supplementary Figure 1. Identification of lymphocyte and monocyte subsets in healthy donors and COVID-19 patients.	44
Supplementary Figure 2. Representative images of inflammasome speck formation in COVID-19 patient monocytes.	45
Supplementary Figure 3. Effect of anti-Spike monoclonal antibodies or pooled COVID plasma on <i>in vitro</i> infection of LPS-activated healthy donor purified monocytes with icSARS-CoV-2-mNG.	46

Acknowledgements

First, I would like to thank all members of the Lieberman lab for welcoming me to the lab and helping me throughout my time here. I have learned a lot from each of you, and I'm a better scientist for it. I would especially like to thank Dr. Ângela Crespo and Dr. Caroline Junqueira, whose mentorship and support have made a challenging year of research amidst a global pandemic a fun and educational experience that I will always hold dear. They taught me all the techniques I used throughout my thesis, and they both contributed heavily to the data presented. I also thank our collaborators from outside the Lieberman lab who were instrumental in obtaining clinical data and human samples; especially Dr. Shahin Ranjbar who performed the *ex vivo* SARS-CoV-2 infection studies. Of course, I also thank Dr. Judy Lieberman for inviting me to join her lab and providing me with thoughtful, helpful guidance throughout my thesis research. Our many conversations made me rethink how I approach science, and your support and guidance were instrumental in my ability to move on to MD/PhD training next year.

Outside the Lieberman lab, I also thank Dr. Shiv Pillai, Dr. Michael Carroll, Selina Sarmiento, and Faith Crisley for their support throughout the master's program. My two years with this program have given me a new appreciation for immunology and basic science research, and I know the skills and experiences I acquired here will stay with me throughout the rest of my training as a physician-scientist. I also thank the entire class of 2021, especially Ksenia Morozova and Shivan Lala, whose friendship helped me through challenging days in lab, all while living through a global pandemic.

Finally, I would like to thank my family. None of this would have been possible without the love and support of my parents, my grandparents, and my sister. They believed in me when I didn't and have supported me unconditionally my entire life. If it weren't for my family, I don't

know where I would be today, but it wouldn't be at Harvard preparing to move on to medical school. Thank you for everything you have done for me and everything you have given up so I could chase my dreams. I wasn't always sure I would get there, but you all were, and for that I can't thank you enough.

This thesis is dedicated to my younger sister, Hannah. You have had to go through so much more than any kid should, and so much more than I ever had to. And yet, through it all you've never given up and you've tackled every obstacle just like the last. Seeing what you had to go through to find a diagnosis, and what you still go through to find a treatment, is part of what makes me want to be a physician-scientist.

Chapter 1: Background

1.1 Introduction

Inflammation and cell death are normal processes that prevent the spread of infection, but when uncontrolled, inflammation and rampant cell death can have drastic consequences for the host. Cell death is often thought of as a programmed, non-inflammatory sequence of events that leads to apoptosis – a process that occurs throughout the body, throughout the day¹. However, inflammatory forms of cell death such as necroptosis and pyroptosis can occur during infection or sterile damage and can lead to local and systemic inflammation through the release of intracellular inflammatory mediators such as IL-1 family cytokines and lactate dehydrogenase (LDH)². Pyroptosis was coined in 2001 to describe an inflammatory form of programmed cell death that was differentiated from necrosis by the active involvement of caspases and from apoptosis by the release of intracellular inflammatory components that could alert the immune system to an infection or other disruption of cellular homeostasis³. These differing forms of cell death can have drastic consequences for human health, and have been shown to be involved in infection, autoimmune disease, and cancer⁴.

Most recently, it has been hypothesized that cell death may play a role in the pathogenesis of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) – the causative agent of the coronavirus-disease 2019 (COVID-19) pandemic that has caused more than 100 million infections and over 2 million deaths worldwide as of March 1st, 2021⁵. A hallmark of severe COVID-19 is an overly exuberant inflammatory response, including CRS, akin to that seen during other hyperinflammatory disease states such as sepsis^{6–8}. However, it is unknown how SARS-CoV-2 triggers inflammation. In sepsis, it is well-appreciated that one of the drivers of

CRS and ultimately severe disease is pyroptosis and a dysregulated myeloid cell compartment^{9–11}. This raises the possibility that pyroptosis may also play a role in CRS during SARS-CoV-2 infection.

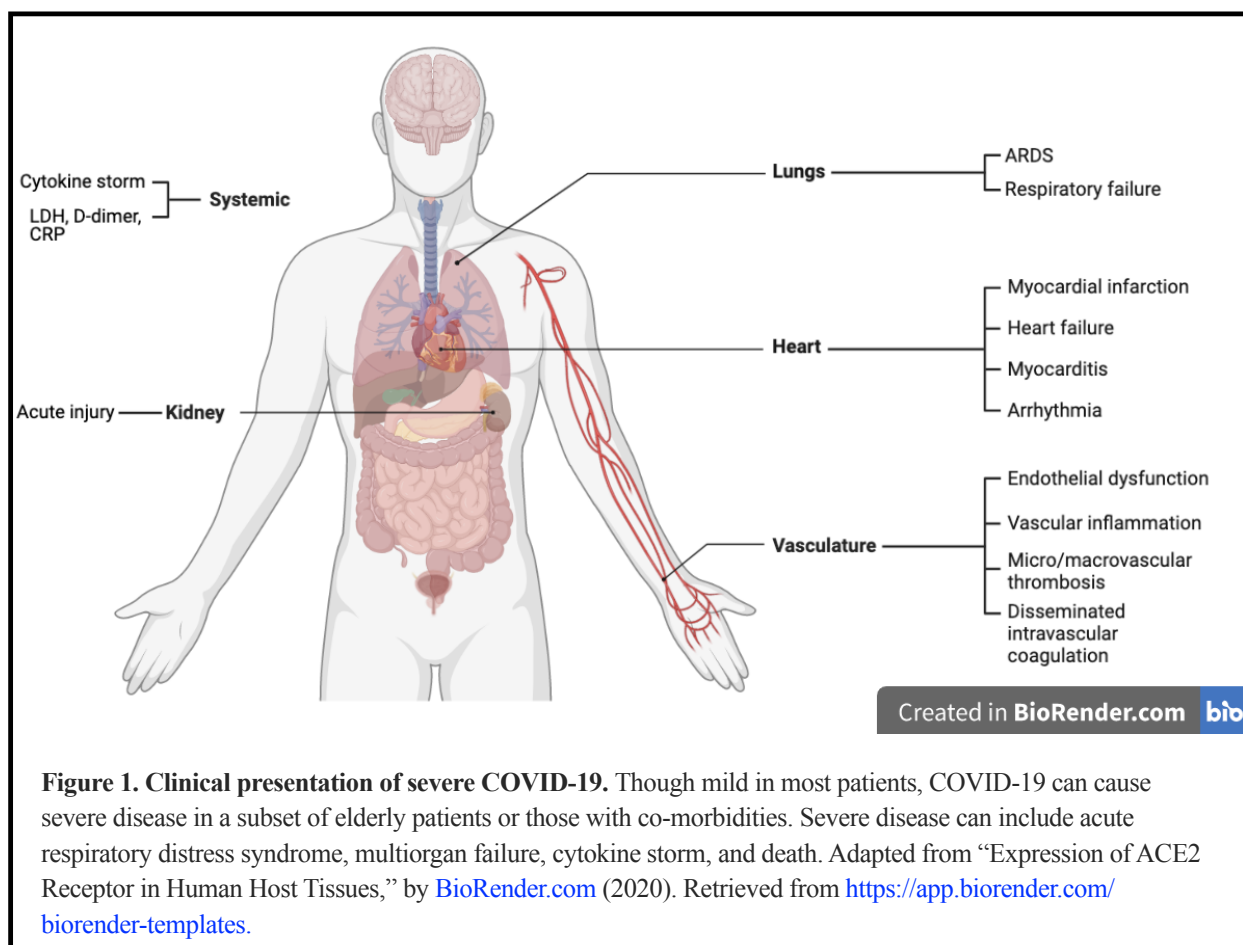
Here, my colleagues and I show that blood monocytes from COVID-19 patients are infected by SARS-CoV-2 and infected monocytes have activated NLRP3 and AIM2 inflammasomes and undergo pyroptosis. Plasma biomarkers of pyroptosis including LDH and the IL-1 family cytokines IL-1 β and IL-18 – whose release are dependent on gasdermin D (GSDMD) pore formation as occurs in pyroptosis – as well as other inflammatory cytokines, chemokines, and growth factors, correlate with development of severe COVID-19. Additionally, purified healthy donor (HD) monocytes can be infected *ex vivo* by SARS-CoV-2, but infection is enhanced by monocyte activation or culture with anti-Spike antibodies or patient plasma. Taken together, these results suggest that SARS-CoV-2 infection of blood monocytes causes pyroptosis, which may be a major driver of CRS during severe COVID-19, and which may provide an opportunity for therapeutic intervention to interrupt hyperinflammation and reduce severe cases of COVID-19.

1.2 The COVID-19 Pandemic

In December 2019, an outbreak of viral pneumonia of unknown etiology was discovered in Wuhan, China¹². Genome sequencing and phylogenetic analysis revealed that the causative agent of this outbreak was a novel *Betacoronavirus*, likely of bat origin, that shared some homology with SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV), and was thus named the 2019-novel coronavirus (2019-nCoV)^{12–14}. As more was learned about this virus and the disease it causes – coronavirus disease 2019 (COVID-19) – it has since been renamed the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2)¹³. Since December 2019,

SARS-CoV-2 has infected more than 100 million people and caused more than 2 million deaths worldwide⁵.

Although most infected people suffer only mild or moderate disease, a subset of mostly older patients or those with underlying health conditions develop severe pneumonia that can progress to hypoxia, acute lung injury, and death¹⁵. Likewise, while most infected children remain asymptomatic or develop mild disease, some develop a widespread inflammatory disease known as multisystem inflammatory syndrome in children (MIS-C)¹⁶. While MIS-C shares some characteristics with Kawasaki disease, it appears to be its own inflammatory condition that differs from the inflammatory pathology seen in severe COVID-19 in adult patients¹⁶. Severe COVID-19 in adult patients is often accompanied by acute respiratory distress syndrome, multiorgan failure including capillary leak, acute kidney injury, and disseminated intravascular coagulation ([Fig. 1](#))¹⁵. A hallmark of clinical deterioration in severe COVID-19 is CRS, or cytokine storm, which is often seen in sepsis and is caused by an uncontrolled inflammatory response to infection⁸. COVID-19 severity is strongly linked to serum levels of pro-inflammatory markers such as interleukin-1 β (IL-1 β), IL-6, tumor necrosis factor- α (TNF α), C-reactive protein (CRP), ferritin, and lactate dehydrogenase (LDH)^{8,17–20}. LDH release is pathognomonic for pyroptosis and is a strong predictor of clinical outcome during severe disease^{21,22}.



Aside from supportive care, the only pharmacological interventions that have shown some limited benefit for treating severe COVID-19 are the nucleoside analog antiviral remdesivir and immune suppression by corticosteroids. Remdesivir inhibits the RNA-dependent RNA polymerase encoded by SARS-CoV-2 that is required for viral replication²³. In a randomized, controlled trial, remdesivir shortened the time to recovery and reduced mortality in patients who were hospitalized with COVID-19 compared with placebo²³. Dexamethasone on the other hand acts on the host immune system rather than the virus itself. Dexamethasone is a glucocorticoid that can suppress the inflammatory response by reducing neutrophil migration and the production of inflammatory mediators, as well as decreasing capillary permeability²⁴. These anti-inflammatory functions make dexamethasone a useful treatment for severe COVID-19, but it is

only effective at reducing mortality in patients who receive respiratory support²⁵, again supporting the idea that severe COVID-19 is a result of an overly exuberant inflammatory response.

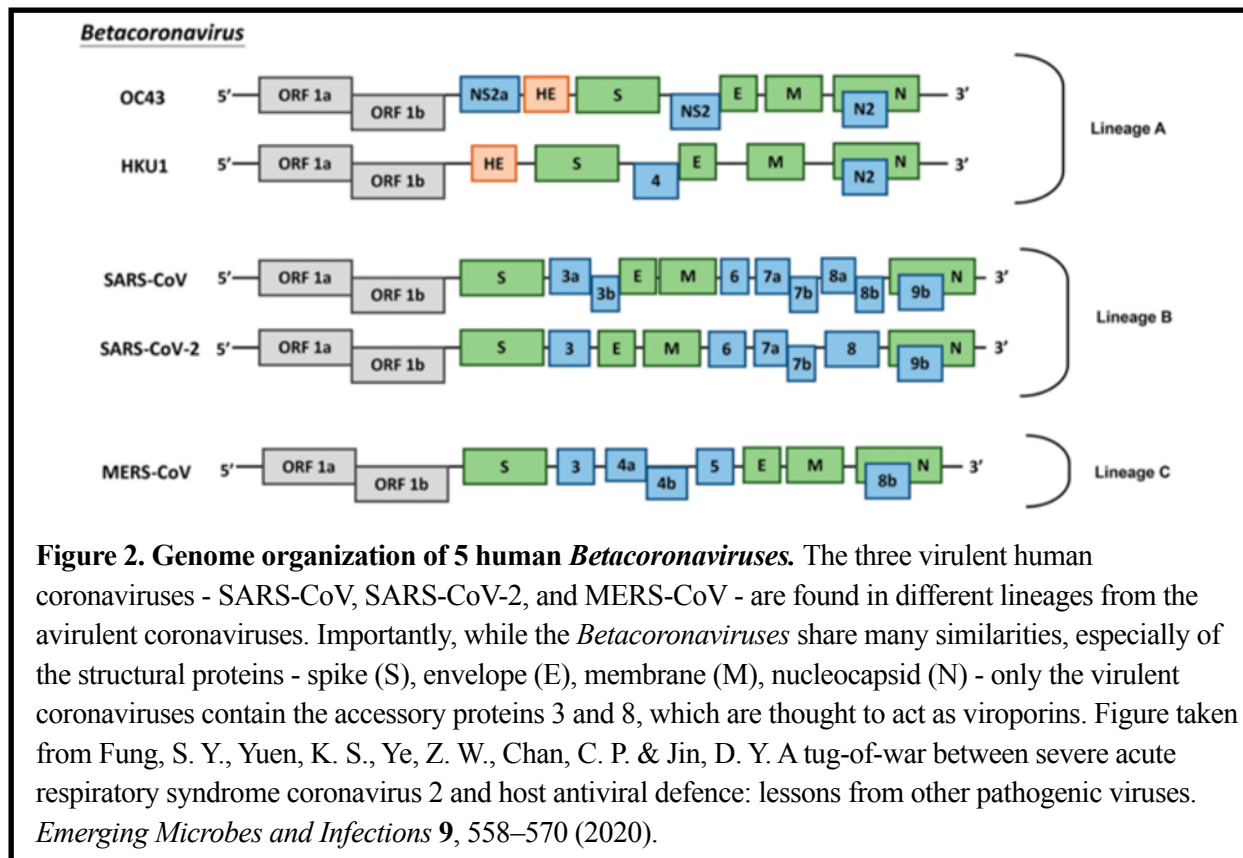
Inhibiting inflammatory cytokines has also been investigated for the treatment of severe COVID-19, with mixed results. Anti-IL-1 β (canakinumab) did not meet its primary endpoint for efficacy in a controlled trial in hospitalized COVID-19 patients²⁶, but an unpublished manuscript of a randomized controlled trial of IL-1RA (anakinra) showed a reduction in respiratory failure and clinical severity in patients with COVID-19 pneumonia²⁷. While IL-1 β antagonism has had mixed results, antagonists of IL-6 have had weak effects on COVID-19^{28,29}. However, the possible utility of IL-1 β antagonism raises the possibility that gasdermin D (GSDMD) antagonism may be therapeutic in COVID-19, since IL-1 β secretion depends on GSDMD pore formation. Two FDA-approved inhibitors of GSDMD – disulfiram (Antabuse)³⁰ and dimethyl fumarate (Tecfidera)³¹ – are worth evaluating in this context. Administration of either of these compounds in mouse models of sepsis – which shares many features, including cytokine release syndrome, with COVID-19 – improved survival and reduced plasma inflammatory cytokine levels.

Two mRNA-based vaccines – one developed by Moderna³² and one by Pfizer-BioNTech³³ – as well as one adenovirus-based vaccine developed by Johnson & Johnson³⁴ have received emergency use authorization (EUA) by the Food and Drug Administration and are being used in the United States and elsewhere to vaccinate at-risk individuals as of March 2021. Several other vaccines have also received approval internationally but have not yet received EUA in the United States. However, better understanding the pathogenesis of SARS-CoV-2 may provide further opportunities for the discovery or development of treatments for severe COVID-19.

1.3 Previous SARS-like coronaviruses

In February 2003, an outbreak of severe acute respiratory syndrome caused by a coronavirus was identified in China. The outbreak ultimately spread to 29 countries and infected over 8,000 people, causing nearly 800 deaths before being contained in July 2003³⁵. Nine years later, an even more deadly coronavirus was identified in Saudi Arabia. This new virus, termed Middle East Respiratory Syndrome Coronavirus (MERS-CoV), continues to infect susceptible individuals in the Arabian Peninsula with a worrying case-fatality rate of 30-40%³⁶. Like SARS-CoV-2, both SARS-CoV and MERS-CoV are *Betacoronaviruses* that bear striking similarity to bat coronaviruses³⁷. While all three of these coronaviruses are distinct etiologic agents, they share some structural similarities like the external spike glycoprotein from which they get the name coronavirus – *corona* being the Latin word for crown³⁷. However, SARS-CoV-2 seems to spread more efficiently – likely driven by asymptomatic or pre-symptomatic infection – but generally causes less severe disease than SARS-CoV or MERS-CoV^{37,38}.

The similarities between SARS-CoV, MERS-CoV, and SARS-CoV-2 extend beyond the spike protein. Amongst other proteins, all three of these viruses contain open reading frames 3, 8, and E³⁷. 3 and 8 are accessory proteins, while E is an important envelope protein. Interestingly, while E is found in one form or another in many *Betacoronaviruses*, 3 and 8 are only found in virulent coronaviruses like SARS-CoV, MERS-CoV, and SARS-CoV-2 (Fig. 2)³⁷.



While less is known about MERS-CoV, many studies have been done to better understand the pathogenesis of SARS-CoV and the role of its encoded proteins. Several studies have shown that SARS-CoV and -CoV-2 proteins 3a, 8, and E are viroporins, or viral proteins with ion channel activities^{39–43}. Since changes in intracellular ion concentrations are one possible activating signal for inflammasomes such as NLRP3⁴, this brings up the interesting possibility that viroporins may be able to activate inflammasomes and induce cell death via changes in ion homeostasis. Indeed, SARS-CoV proteins 3a, 8, and E can each activate the NLRP3 inflammasome through mechanisms that may include alterations in intracellular potassium and calcium concentrations when ectopically expressed^{41,44,45}.

Interestingly, bats, the natural zoonotic hosts of SARS-CoV and SARS-CoV-2, have a dampened NLRP3 response to viral infections including MERS-CoV, which may explain their

ability to harbor high viral loads⁴⁶. In addition to suppression of NLRP3, bats also lack the DNA-sensor AIM2, and have dampened downstream caspase-1 activity following inflammasome activation⁴⁷. While inconclusive, this adds credence to the idea that severe disease following virulent coronavirus infection may be caused, in part, by inflammasome activation and pyroptosis.

1.4 The SARS-CoV-2 Genome

Coronaviruses have the largest genome of all RNA viruses⁴⁸, and many of the virulent coronaviruses such as SARS-CoV, MERS-CoV, and SARS-CoV-2 share striking similarities. There are three primary types of proteins encoded by SARS-CoV-2 – structural proteins, nonstructural proteins, and accessory proteins³⁷. The structural proteins encoded by SARS-CoV-2 are the spike protein (S), envelope protein (E), membrane protein (M), and nucleocapsid protein (N)([Fig. 2](#))^{48,49}. These structural proteins are conserved across coronaviruses and are required for viral entry and replication³⁷. SARS-CoV-2 entry into host cells depends on S protein binding to the human receptor angiotensin-converting enzyme-2 (ACE2)⁵⁰. Thus, S is an attractive target for preventing infection; indeed, multiple vaccines currently in use around the world target the S protein⁵⁰ and several pharmacologic interventions are in trials such as the use of recombinant human ACE2⁵¹ that S will preferentially bind to over endogenous ACE2 present on susceptible host cells.

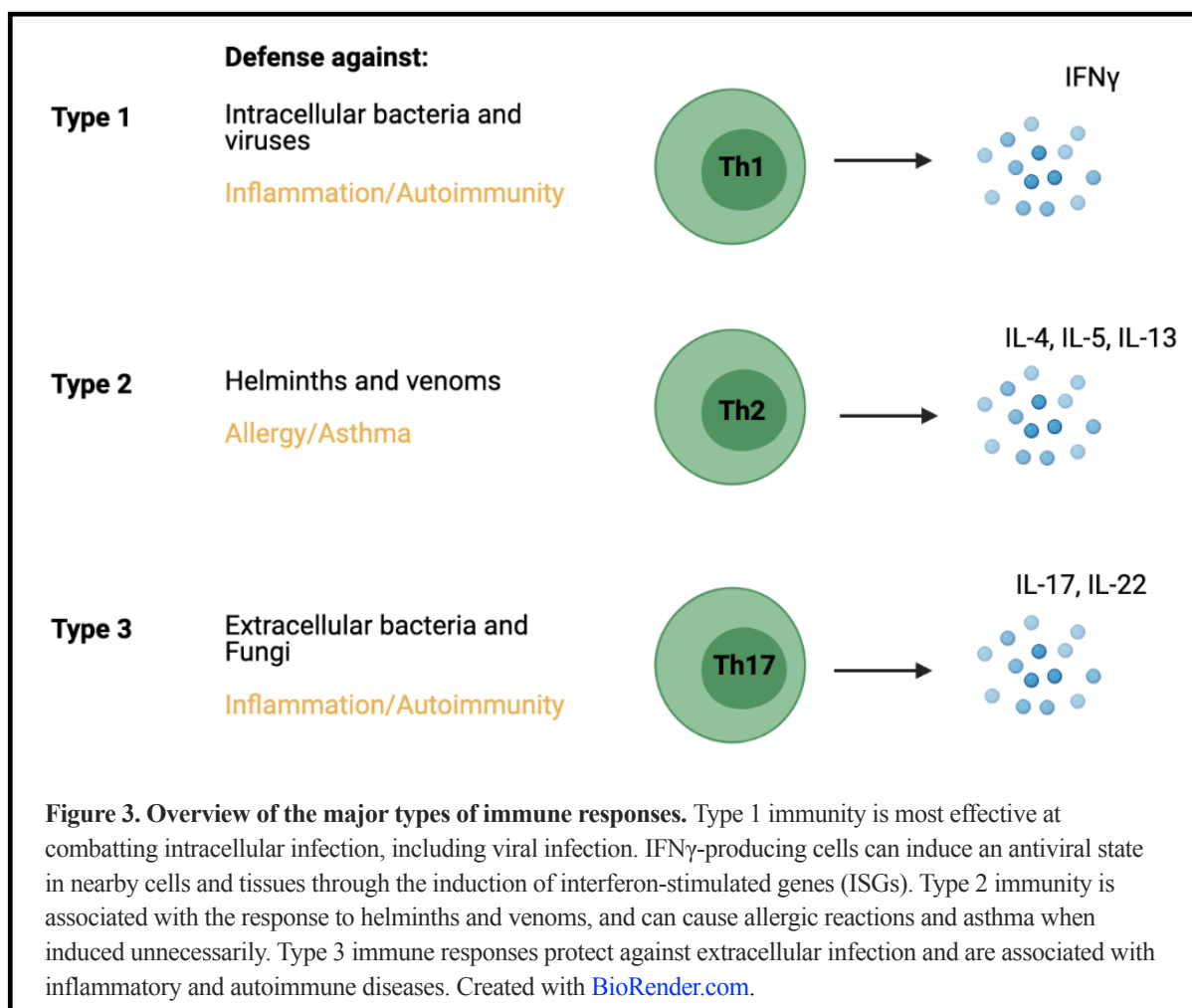
Additionally, there are 16 nonstructural proteins produced via cleavage of the two polypeptides produced by translation of ORF1a and ORF1b ([Fig. 2](#))^{48,49}. These nonstructural proteins have a variety of functions including protease activity and producing an RNA-dependent RNA-polymerase that is required for viral replication⁴⁸. In addition to structural and nonstructural proteins, SARS-CoV-2 also encodes a variety of accessory proteins. While

predictions of the number of these proteins can vary, it is likely that SARS-CoV-2 has at least six accessory proteins, including 3a, 6, 7a, 7b, 8, and 10^{48,49}. Importantly, 3a and 8 are encoded by virulent coronaviruses including SARS-CoV and MERS-CoV, but not avirulent coronaviruses (Fig. 2)³⁷. As discussed previously, when ectopically expressed these accessory proteins form viroporins that can activate the NLRP3 inflammasome by K⁺ efflux²⁰. This suggests that ORFs 3a and 8 might play a role in the virulence of SARS-like coronaviruses, and that this virulence may be driven in part by inflammasome activation and pyroptosis.

1.5 The Antiviral Response

The response to infection is a highly coordinated, finely tuned immune system reaction that requires both local and systemic responses. How the immune system responds to infection depends on what kind of pathogen it is – viral, bacterial, or fungal – and where in the body the infection occurs. Extracellular infections are primarily responded to through type 2 and type 3 immune responses (Fig. 3). A type 2 immune response is marked by Th2 cell involvement and IgE production and is also a mediator of allergic reactions⁵². Type 3 immune responses primarily involve Th17 cells that recruit and activate neutrophils and mononuclear phagocytes to protect against extracellular bacteria and fungi but can also mediate some autoimmune diseases⁵². While these inflammatory responses are useful for combating extracellular infection, responding to a viral infection requires a type 1 immune response involving Th1 cells and other interferon (IFN)-producing cells (Fig. 3)⁵². In the context of viral infection, the production of type I and type III interferons and the induction of the antiviral state in epithelial cells is preferred over an inflammatory response as is seen during extracellular infection. The master regulators of IFN production are NF- κ B and IRF3/7, which can be activated by a variety of stimuli including IL-1 β , TNF α , and recognition of infection by pattern recognition receptors (PRRs)^{20,53}. Once

activated, NF- κ B acts as a transcription factor for the production of type I IFNs, which when released can activate an antiviral state in nearby cells to prevent further infection and viral replication. Type I and III IFNs both induce interferon-stimulated genes (ISGs) that establish a state of viral resistance⁵³. If this interferon response is replaced by a more inflammatory response, excess immune-mediated damage to the host may occur without a concomitant increase in antiviral immunity – as seems to be occurring in severe COVID-19.



Multiple reports have suggested that in COVID-19 there is a dysregulated immune response that favors inflammation over antiviral IFN responses. Indeed, type I and III IFNs are

rarely detected in COVID-19 patient plasma, and IFN induction is depressed following infection with virulent coronaviruses compared to the avirulent coronavirus HCoV-229E⁵³. Further, the virulent coronaviruses SARS-CoV, MERS-CoV, and SARS-CoV-2 have evolved mechanisms to dampen the IFN response. A primary mechanism of coronavirus evasion of innate immunity is replication within double membrane vesicles, which shields dsRNA replication intermediates from recognition by cytoplasmic PRRs, such as AIM2⁵³. SARS-CoV Nsp14 and Nsp16 can modify viral RNA to mimic the 5' cap that PRRs such as RIG-I use to distinguish viral RNA from host RNA⁵³. Coronaviruses can also inhibit IFN production via M, N, and Nsp3 proteins, which all interfere with IRF3 nuclear translocation and function⁵³. IRF3 is a major transcription factor for IFN genes, so blocking IRF3 function limits the production of type I and III IFNs in infected cells. SARS-CoV Orf6 and Nsp1 both block IFN signaling by inhibiting nuclear translocation and phosphorylation of STAT1, respectively⁵³. Since STAT1 is a master regulator of ISGs, this prevents the induction of interferon-stimulated genes. RNase L is one such ISG that cleaves single-stranded viral RNA in the cytosol. MERS-CoV ORF4b prevents RNase L activation⁵³.

Additionally, there is some evidence that inborn mutations in IFN induction and signaling, and autoantibodies against type I IFNs may play a role in susceptibility to severe COVID-19⁵⁴. The kinetics of IFN production may also determine disease severity. An early IFN response is more likely to control viral load and inflammation; indeed, several studies have shown that early treatment with IFN β 1a improved clinical outcomes and decreased recovery time compared to placebo or antiviral therapy alone^{54–56}. In contrast, patients with a delayed or deficient IFN response may be more likely to progress to severe disease, though this must be confirmed in clinical studies⁵⁴. Instead of a strong IFN response and induction of the antiviral

state, COVID-19 patients show signs of an inflammatory response, marked by increases in the plasma inflammatory cytokines IL-18, IL-6, and TNF α ^{20,53}. When left uncontrolled, this inflammatory response can lead to damaging immunopathology.

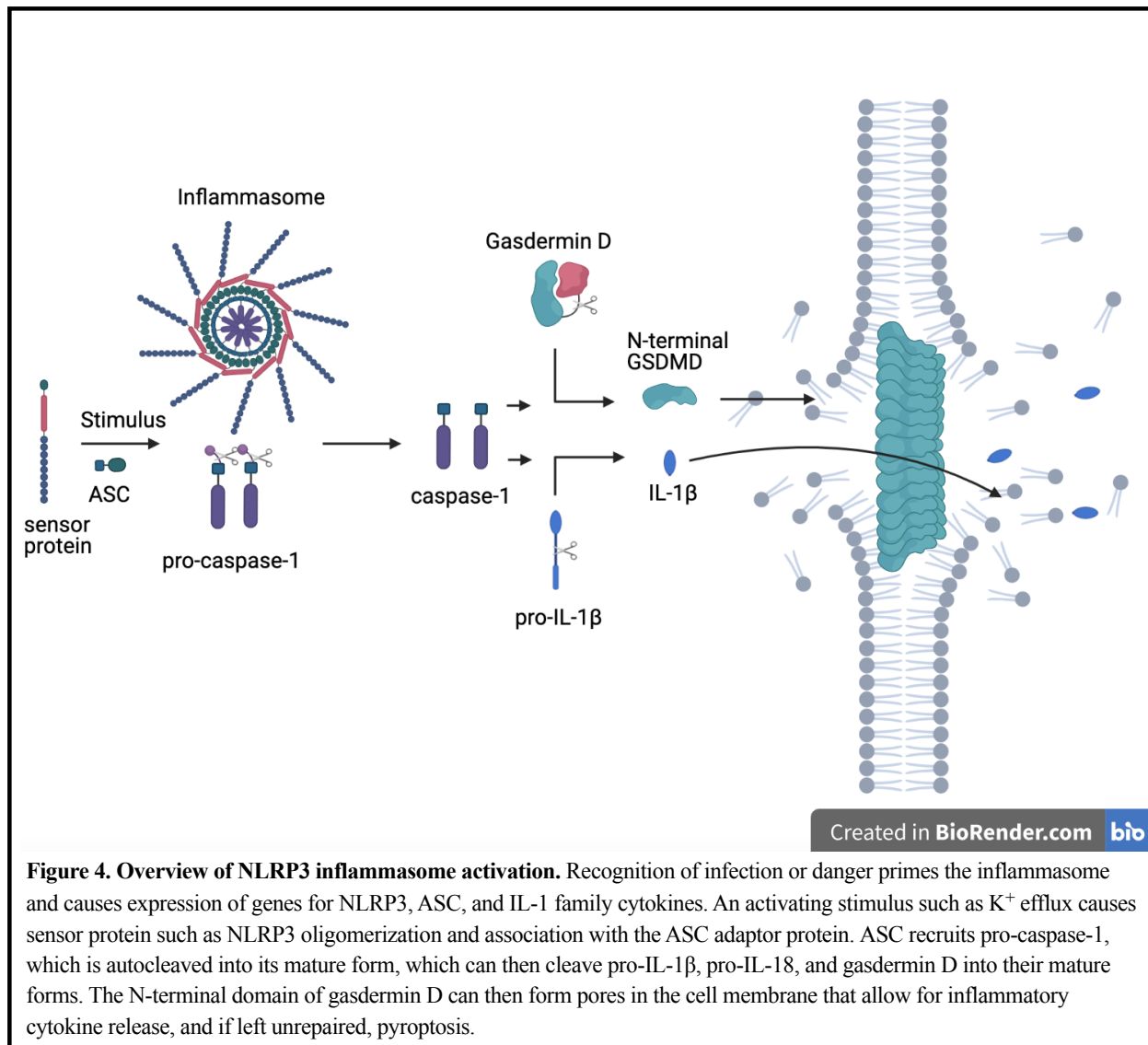
1.6 Inflammatory Cell Death

A major driver of severe clinical disease and CRS during sepsis is pyroptosis of myeloid cells. Pyroptosis is a form of inflammatory cell death that relies on membrane damage by pore-forming proteins called gasdermins. In contrast to apoptosis, pyroptosis is inflammatory because cell membranes are damaged during pyroptotic cell death, allowing intracellular inflammatory mediators such as IL-1 β and IL-18 to be released²². IL-1 β is one of the most potent inflammatory cytokines, and as such is tightly regulated by its antagonist, IL-1RA^{57,58}. IL-18 is a prominent inducer of IFN γ and is increased in a number of autoimmune and inflammatory diseases, including systemic lupus erythematosus, rheumatoid arthritis, and type 1 diabetes⁵⁸. LDH is pathognomonic of pyroptosis²² and is increased in the plasma in a number of systemic inflammatory conditions, including sepsis and COVID-19²¹. Thus, while apoptosis and pyroptosis are both forms of programmed cell death that rely on the activity of caspases, pyroptosis causes local and systemic inflammation while apoptosis does not.

Pyroptosis occurs in response to infection or tissue damage and is a means of informing the immune system of a problem. One of the major cell types that undergo pyroptosis is myeloid cells – the sentinel cells of the innate immune system responsible for sounding the alarm for infection. There are 27 potential human canonical inflammasome sensors²², although the most well studied is NLRP3, which can detect a diverse range of stimuli such as K⁺ efflux⁴. Upon activation, these sensor proteins oligomerize and form cytosolic specks that can be observed by microscopy or imaging flow cytometry. Sensor proteins associate with adaptor proteins such as

apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC)(Fig. 4)⁴. ASC contains two death domains – PYD and CARD – that form homotypic interactions with the sensor proteins and pro-caspase-1, respectively⁴. Once ASC and a sensor such as NLRP3 form a macromolecular complex (called the inflammasome), pro-caspase-1 is recruited via CARD-CARD interactions between ASC and pro-caspase-1 (Fig. 4)⁴. This interaction facilitates autoproteolytic cleavage of pro-caspase-1 into active caspase-1⁵⁹, which can then cleave pro-IL-1 β , pro-IL-18, and GSDMD into their active forms⁴. Once cleaved, the N-terminal portion of GSDMD traffics to the cell membrane and forms pores that release intracellular inflammatory mediators such as the active forms of IL-1 β and IL-18, as well as ATP and HMGB1 (Fig. 4)^{2,22}. LDH is too large to fit through GSDMD pores, but is released when cells lyse²². Since IL-1 family cytokines lack a signal peptide, membrane pore formation is the primary mechanism for cytokine release⁵⁸. Left unrepaired, these pores cause cell death, or pyroptosis.

Pyroptosis can occur in other cell types, including epithelial cells²². The general sequence of events remains the same, though depending on the cell-type and the stimulus, different inflammasomes may be activated. Importantly, pyroptosis requires two signals – one to prime the cell, and one to activate the inflammasome (Fig. 4). The priming signal, such as the recognition of lipopolysaccharide by TLR4, leads to the transcription of inflammatory genes such as pro-IL-1 β and pro-IL-18²². A second signal is then required, such as extracellular ATP, to activate the inflammasome and allow caspase-1 activation²².



1.7 Conclusions

While most COVID-19 patients have only mild disease, a small subset of mostly elderly patients or those with comorbidities develop severe disease that can include acute respiratory distress syndrome, CRS, multiorgan failure, and death^{8,15}. A hallmark of this severe disease state is an overly exuberant inflammatory response; however, it is unknown how SARS-CoV-2 infection triggers such inflammation. Through analysis of COVID-19 patient and healthy donor monocytes, and *ex vivo* SARS-CoV-2 infection of healthy donor monocytes, my co-workers and

I investigated how SARS-CoV-2 activates the innate immune system and triggers inflammatory cytokine production.

In this thesis, we show that inflammatory cytokines, growth factors, and markers of pyroptosis are found in COVID-19 patient plasma and correlate with development of severe disease. We found that COVID-19 patient blood monocytes are productively infected by SARS-CoV-2 and undergo pyroptosis. These infected monocytes contain activated NLRP3 and AIM2 inflammasomes, active caspase-1, cleaved GSDMD, and processed IL-1 β , indicating that canonical inflammasome activation in infected blood monocytes leads to pyroptosis and cytokine release. Additionally, purified monocytes from healthy donors can be infected by SARS-CoV-2 *ex vivo*, and this infection is enhanced by activation by LPS, or co-culture with anti-Spike antibodies or patient plasma. Infection is abrogated by IgG depletion from patient plasma. These results suggest a role for antibody-dependent uptake of viral particles, which leads to inflammasome activation, pyroptosis, and cytokine release, which may drive severe disease in some patients. However, it is unclear how the NLRP3 and AIM2 inflammasomes are activated in patient monocytes or if other inflammasomes are activated.

Chapter 2: Results

Adapted from Junqueira, C. *et al.* SARS-CoV-2 infects blood monocytes to activate NLRP3 and AIM2 inflammasomes, pyroptosis and cytokine release. *medRxiv* (2021).
doi:10.1101/2021.03.06.21252796

2.1 Introduction

Although inflammation and CRS are hallmarks of clinical deterioration in COVID-19 patients, how SARS-CoV-2 triggers inflammation is not well understood. Because inflammasome activation in myeloid cells is a major mediator of inflammatory cytokine processing and release²², and because plasma levels of inflammatory cytokines are linked to COVID-19 disease severity^{17,20}, my co-workers and I examined blood from SARS-CoV-2 infected donors for signs of inflammasome activation. We found that circulating monocytes from COVID-19 patients are infected by SARS-CoV-2 and undergo pyroptosis following NLRP3 and AIM2 inflammasome activation. Biomarkers of pyroptosis in the blood, such as LDH, IL-18, and IL-1RA, correlate with disease severity. Infection of healthy donor monocytes by SARS-CoV-2 was enhanced in the presence of anti-Spike antibodies or patient plasma.

2.2 Materials and Methods

2.2.1 Cell Lines and Reagents

Reagent or Resource	Source	Identifier (clone, Cat. #)	
Antibodies			
Flow Cytometry			Dilution
Mouse anti-human PerCP-Cy5.5 CD4	BioLegend	Clone SK3; Cat. # 344608	1:200

Mouse anti-human PE-Cy7 CD14	BioLegend	Clone HCD14; Cat. # 325618	1:200
Mouse anti-human APC CD16	BD Biosciences	Clone B73.1; Cat. # 561304	1:200
Mouse anti-human PE-CF594 CD16	BD Biosciences	Clone 3G8; Cat. # 562320	1:200
Mouse anti-human AlexaFluor™ 488 CD19	BioLegend	Clone SJ25C1; Cat. # 363038	1:200
Mouse anti-human APC-Cy7 CD19	BioLegend	Clone SJ25C1; Cat. # 363010	1:200
Mouse anti-human Brilliant Violet™ 650 CD19	BioLegend	Clone HIB19; Cat. # 302237	1:100
Mouse anti-human APC-Cy7 CD8	BioLegend	Clone SK1; Cat. # 344714	1:200
Mouse anti-human Brilliant Violet™ 421 CD3	BioLegend	Clone UCHT1; Cat. # 300434	1:200
Mouse anti-human APC-Cy7 CD3	BioLegend	Clone UCHT1; Cat. # 300426	1:200
Mouse anti-human APC-Cy7 CD56	BioLegend	Clone HCD56; Cat. # 318332	1:100
Mouse anti-human Brilliant UltraViolet™ 737 CD56	BD Biosciences	Clone NCAM16.2; Cat. # 349105	1:200
Mouse anti-human AlexaFluor™ 488 HLA-DR	BioLegend	Clone G46-6; Cat.# 307620	1:200
Mouse anti-human Brilliant UltraViolet™ 395 HLA-DR	BD Biosciences	Clone G46-6; Cat. # 564040	1:200
Mouse anti-human PerCP-Cy5.5 CD123	BD Biosciences	Clone 7G3; Cat. # 558714	1:100
Mouse anti-human Brilliant Violet™ 421 CD11c	BD Biosciences	Clone B-LY6; Cat. # 562561	1:200
Mouse anti-human purified CD32	StemCell Technologies	Clone IV.3; Cat. # 60012	1:100
Goat anti-human/mouse/rat/hamster purified ACE2	R&D Systems	Cat # AF933	1:100
Normal Goat IgG Control	R&D Systems	Cat # AB108C	1:100

Mouse anti-human APC CD147	BioLegend	Clone HIM6; Cat # 306214	1:100
Imaging Flow Cytometry, Immunofluorescence and Western blot			
Mouse anti-dsRNA	SCICONS	Clone J2; Cat. # 1001020	1:500
Rabbit purified anti-SARS-CoV-2 Nucleocapsid	GeneTex	Polyclonal; Cat. # GTX135357	1:500
Mouse anti-human purified ASC	Sigma	Clone 2EI-7; Cat. # 04-147	1:200
Rabbit anti-human purified ASC	Santa Cruz	Polyclonal; Cat. # sc-22514-R	1:200
Goat anti-human purified NLRP3	Abcam	Cat #ab4207	1:200
Mouse anti-human purified AIM2	Abcam	Clone 3C4G11; Cat. # ab204995	1:200
Rabbit anti-human purified MEFV	Proteintech	Polyclonal; Cat. # 24280-1-AP	1:200
Mouse anti-human GSDMD antibody	Lieberman lab	Hybridoma (ref 55)	1:200
Rabbit anti-human GSDMD C-terminal	Abcam	Cat. # ab210070	1:1000
Rabbit anti-human COX-IV	Cell Signaling	Clone 3E11; Cat. # 4850S	1:1000
Mouse anti- β -actin	DSHB	Cat. # JLA-20	1:1000
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher	Polyclonal; Cat. # A-21206	1:1000
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	ThermoFisher	Polyclonal; Cat. # A-31573	1:1000
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	ThermoFisher	Polyclonal; Cat. # A-31571	1:1000
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher	Polyclonal; Cat. # A-21202	1:1000

Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546	ThermoFisher	Polyclonal; Cat. # A- 11056	1:1000
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher	Polyclonal; Cat. # A- 11055	1:1000
Amersham ECL Mouse IgG, HRP- linked whole Ab (from sheep)	Cytiva Life sciences	Cat.# NA931-1ML	1:5000
Amersham ECL Rabbit IgG, HRP- linked whole Ab (from donkey)	Cytiva Life sciences	Cat. # NA934-1ML	1:5000
Functional Assays			
Human IgG1	Johnatan Abraham, Harvard Medical School	Clone mAb114	10 µg/ml
Human anti-SARS-CoV-2 Spike protein, neutralizing	Johnatan Abraham, Harvard Medical School	Clone C1A-B12	10 µg/ml
Human anti-SARS-CoV-2 Spike protein, non-neutralizing	Johnatan Abraham, Harvard Medical School	Clone C1A-H12	10 µg/ml
Reagents, Peptides and recombinant proteins			
RPMI 1640	Corning	Cat. # 15-040-CV	
PBS	Gibco	Cat. # 14190250	
Fetal Calf Serum	X&Y Cell Culture	Cat. # FBS-500-HI	
Human AB Serum	Gemini Bio- Products	Cat. # 100-512	
Streptomycin sulfate salt	Sigma	Cat. # S9137-100G	
Penicillin G potassium salt	Sigma	Cat. # P7794-100MU	
4% Paraformaldehyde in PBS	Santa Cruz	Cat. # 30525-89-4	
Ficoll® Paque Plus	GE Healthcare	Cat. # GE17-1440-02	

Red Blood Cell Lysing Buffer Hybri-Max	Sigma	Cat. # R7757-100ML	
Triton-X 100	Sigma	Cat. # X100-500ML	
Rho Inhibitor I	Cytoskeleton	Cat. # CT03	
LPS	Sigma	Cat. # L6529-1MG	
Nigericin	Sigma	Cat. # N7143-5MG	
Nigericin	Invivogen	Cat. # tlrl-nig-5	
DAPI	Thermo Scientific	Cat. # 62248	1:1000
Airvol ® 205 Polyvinyl alcohol	Air Products and Chemicals	N/A	
Chloroform	Fisher Scientific	Cat. # BP1145-1	
2-Propanol	Fisher Scientific	Cat. # A451-1	
Ethanol	Koptec	Cat. # V1016	
Nuclease-Free water	Invitrogen	Cat. # AM9932	
Critical Commercial Assays			
Annexin V PE	ThermoFisher	Cat. # A35111	1:200
Annexin V PE	BD Biosciences	Cat. # 560930	1:100
Annexin V Buffer 10x	BD Biosciences	Cat. # 556454	
Zombie Yellow Fixable Viability Dye	BioLegend	Cat. # 423104	1:200
CD14 MicroBeads, human	Miltenyi Biotec	Cat. # 130-050-201	
FAM FLICA™ Caspase-1 Kit	BioRad	Cat. # ICT097	
Human Gasdermin D ELISA Kit	MyBiosource	Cat. # MBS2031915	
LEGEND MAX SARS-CoV-2 Spike RBD human IgG ELISA Kit	BioLegend	Cat. # 447707	
CytoTox 96® Non-Radioactive Cytotoxicity Assay	Promega	Cat. # G1780	

Human Magnetic Luminex ® Assays, Premixed Multiplex	R&D Systems	Cat. # LXSAHM-22	
EDTA tubes for blood collection	BD Life Sciences	Cat. #366643	
Trizol	ThermoFisher	Cat. # 15596026	
TURBO DNA-free™ Kit	ThermoFisher	Cat. # AM1907	
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems	Cat. #4368813	
Sso Fast™ EvaGreen® Supermix	BioRad	Cat. #1725204	
Protein A/G agarose resin	ThermoFisher	Cat. # 20423	
Control agarose resin	ThermoFisher	Cat. #26150	
Primers		Sequences	
<i>ACE2</i> primers	Integrated DNA Technologies	Forward: 5' ATACTGTGACCCCGCATCTC 3'; Reverse: 5' CTGATTTTCCAAGCCTCAGC 3'	
β -actin (<i>ACTB</i>) primers	Integrated DNA Technologies	Forward: 5' AAGGCCAACC GCGAGAAGAT 3'; Reverse: 5'ACAGCCTGGATAGCAACGTAC A 3'	
CD147 (<i>BSG</i>) primers	Integrated DNA Technologies	Forward: 5' CAGAGTGAAGGCTGTGAAGTCG 3'; Reverse: 5' TGCGAGGAACTCACGAAGAAC 3'	

2.2.2 Human subjects recruitment. *Fresh PBMCs and plasma cohort.* The study was approved by the Investigation Review Boards of Boston Children's Hospital and Massachusetts General Hospital (MGH), and all enrolled patients signed an informed consent. 35 patients 18 years or older with clinical symptoms suggestive of COVID-19 infection were enrolled at the time of presentation to the MGH emergency department (ED) from 7/9/20 to 01/12/21. A 10-ml EDTA

blood sample was transported to Boston Children's Hospital and processed within 2 h of collection. Only samples from patients with qRT-PCR-verified SARS-CoV-2 infection were included in the study. Healthy donor (HD) samples were processed and analyzed in parallel with patient samples.

Frozen plasma cohort. 60 patients 18 yr or older with clinical symptoms suggestive of COVID-19 infection were enrolled in the MGH ED from 3/15/20 to 4/15/20 with an IRB-approved waiver of informed consent. Enrolled patients had at least one of the following: (i) tachypnea ≥ 22 breaths per minute, (ii) oxygen saturation $\leq 92\%$ on room air, (iii) requirement for supplemental oxygen, or (iv) positive-pressure ventilation. A 10-ml EDTA tube was obtained with the initial clinical blood draw in the ED (n=60). Blood was also obtained on days 3 (n=42) and 7 (n=35) if the patient was hospitalized on those dates. Clinical course was followed for 28 d post-enrollment or until hospital discharge if after 28 d. SARS-CoV-2-confirmed patients (by qRT-PCR) were assigned a maximum acuity score (A1-A5) (A1 – died, A2 – required mechanical ventilation, A3 – hospitalized requiring supplemental oxygen, A4 – hospitalized but not requiring supplemental oxygen, A5 – discharged and not requiring hospitalization)^{60,61}. Patients were grouped based on their worst acuity score over 28 d and divided into three groups for comparison (A1 and A2, severe disease; A3, moderate disease; A4 and A5, mild disease). Only 1 patient was in A4; therefore, most mild patients represent those that were discharged directly from the ED and thus have only a day 0 sample. CRP and D-dimer values were grouped in the following categories: CRP (mg/L) 1 = 0-19.9, 2 = 20-59.0, 3 = 60-99.9, 4 = 100-179, 5 = 180+; D-dimer (ng/mL) 1 = 0-499, 2 = 500-999, 3 = 1000-1999, 4 = 2000-3999, 5 = 4000+.

2.2.3 Plasma, PBMC, and monocyte isolation. Samples were processed using recommended safety precautions in a BSL-2+ facility. Blood tubes were centrifuged at 2000 rpm for 10 min to

separate plasma from blood cells. Plasma was collected to a new tube and incubated or not with 1% Triton X-100 for 1 h on ice before aliquoting and freezing at -80°C. Blood cells were resuspended in PBS and layered over Ficoll for density centrifugation. PBMC were collected from the interface and subjected to red blood cell lysis (if necessary) with Red Blood Cell Lysing Buffer Hybri-Max for 5 min on ice, followed by quenching with RPMI medium supplemented with 10% FBS and 1% Penicillin/Streptomycin. PBMC were washed once more with RPMI and one fraction was stained for flow cytometry, while the remaining cells were used for monocyte purification by magnetic separation using CD14⁺ magnetic beads. Sources of reagents are described in **2.2.1**.

2.2.4 Luminex, ELISA, and LDH activity assays. IL-1 β , IL-1RA, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12, IL-13, IL-17, IL-18, IL-21, IL-23, CCL3, CCL7, CCL9, CXCL10, G-CSF, TNF- α , IFN- β and IFN- γ were measured in plasma samples using a custom Luminex assay (R&D Systems), following the manufacturer's instructions. Plates were analyzed using a Luminex MAGPIX Analyzer at the Analytical Instrumentation Core Lab of Boston University. GSDMD was measured in the same samples using the Human GSDMD ELISA kit (MyBiosource) following the manufacturer's instructions, and LDH activity was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega). Results from the latter assays were analyzed using a Biotek Synergy 2 analyzer; GSDMD absorbance was measured at 450 nm and LDH absorbance was measured at 490 nm.

2.2.5 Monocyte treatment and culture. Purified monocytes, and THP-1 cells (ATCC) as controls, were cultured in RPMI + 10% FBS + 1% Pen/Strep for 30 min at 37°C in the presence or absence of 20 μ M nigericin. Following incubation, monocytes were washed and used for

FLICA assay, or fixed for 10 min with 4% PFA, washed twice with PBS + 3% FBS and kept at 4° C in PBS + 3% FBS until imaging flow cytometry or confocal microscopy.

As a positive control for pyrin activation, HD monocytes were cultured overnight with 2 µg/ml C3 Transferase from Clostridium botulinum (Rho I inhibitor), then fixed for 10 min with 4% PFA before staining for imaging flow cytometry (data not shown).

2.2.6 Intracellular staining for imaging flow cytometry and confocal microscopy. Fixed monocytes were permeabilized with 0.1% Triton X-100 for 10 min and washed twice with PBS + 3% FBS. Monocytes were then blocked for 30 min with PBS + 5% FBS, washed twice and then stained with unconjugated primary antibodies for ASC (1:200, mouse or rabbit), NLRP3 (1:200, goat), AIM2 (1:200, mouse), GSDMD (1:200, mouse), pyrin (1:200, rabbit), dsRNA (J2, mouse) (1:500) or SARS-CoV-2 nucleocapsid protein (1:500, rabbit) for 2 h, followed by 3 washes with PBS + 3% FBS. Cells were then stained with secondary antibodies (donkey anti-mouse, rabbit or goat conjugated with AlexaFluor 488, 546 or 647, at 1:1000) for 1 h in PBS + 3% FBS, followed by 3 washes.

For microscopy, cells were then stained with DAPI (1:1000) for 10 min, washed 3 times and cytopun onto glass slides (VWR); sealed using polyvinyl alcohol and 1.5 mm coverslips (VWR). Confocal images were acquired using a Zeiss LSM 800 with 405, 488, 561 and 633 nm lasers (emission filters, 465, 509, 561 and 668 nm, respectively) and a 63x 1.4NA oil immersion objective. Images were processed using Zen Blue 3.2.

For imaging flow cytometry, cells were resuspended in PBS + 3% FBS for analysis. Data were acquired using an ImageStream X MKII (Amnis) with 63x magnification and analyzed using

Ideas software (Amnis). Monocytes were gated on area/aspect ratio. ASC, NLRP3, AIM2 and Pyrin specks were gated and quantified based on fluorophore intensity/max pixels.

2.2.7 Flow cytometry. PBMC were washed and stained for viability with Zombie Yellow in PBS (1:200) for 15 min on ice. Cells were washed with PBS, centrifuged, and then stained with Annexin V PE (1:200) in 1x Annexin Buffer for 15 min on ice. After washing with 1x Annexin V buffer, cells were blocked for 10 min with anti-CD32 (1:100) in PBS + 3% FBS, and then stained for 15 min on ice with a cocktail of antibodies to identify lymphocyte and myeloid cell subsets (all 1:200 except CD19 BV650, CD123 PerCP-Cy5.5 and CD56 APC-Cy7, 1:100). Purified monocytes and an A549 cell line overexpressing ACE2 were blocked with anti-CD32, then stained with primary antibodies for ACE2 (1:100) for 15 min on ice. The secondary anti-goat AF488 was co-incubated with CD14 PE-Cy7 (1:200) and CD147 APC (1:100). After the last wash, cells were resuspended in 2% PFA and kept at 4°C until flow cytometry analysis. In vitro-infected monocytes were fixed and permeabilized with 0.1% Triton X-100, then blocked with PBS + 5% FBS. Cells were stained with primary antibodies for dsRNA (J2, mouse) (1:500), then stained with secondary antibody (donkey anti-mouse conjugated with AlexaFluor 647, at 1:500) and anti-CD14 PE-Cy7. Cells were acquired using a FACS Canto II or LSR II and data were analyzed using FlowJo Version 10.

2.2.8 FLICA assay. Monocytes, cultured or not for 30 min with nigericin, were washed and resuspended in RPMI 10% FBS with FLICA substrate (BioRad FAM-FLICA Caspase-1 kit), and cultured for 1 h at 37° C. Cells were then washed twice with 1X Apoptosis Buffer (from the kit) and fixed with 1x Fixative (from the kit). Cells were kept at 4°C until further staining and analysis.

2.2.9 *In vitro* SARS-CoV-2 infection of healthy donor monocytes. icSARS-CoV-2-mNG (a molecular clone of SARS-CoV-2 expressing Neon Green fluorescent protein) was a gift to AEG from Shi Pei Yong and the World Reference Center for Emerging Viruses and Arboviruses, Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX⁶². The NG fusion protein is only expressed during viral replication. HD monocytes (purified from apheresis leukoreduction collars collected at Brigham and Women's Hospital) were incubated overnight with medium or 100 ng/ml LPS, and then infected with icSARS-CoV-2-mNG (MOI =1) in a BSL-3 facility. The viral inoculum was treated with 10 mg/ml of antibody (isotype control mAb114, anti-Spike C1A-H12, or anti-Spike C1A-B12), or 10% HD or COVID-19 patient pooled plasma (heat inactivated or not; Ig-depleted or not, as indicated) before infection with SARS-CoV-2 for 30 min at room temperature. 100 µl of treated virus was added to monocytes (2×10^6 cells/well) in 48 well plates. Infected cells were incubated at 37°C, 5% CO₂ with gentle shaking every 10 min for 1 h, after which the culture volume was increased to 500 µl with RPMI supplemented with 5% heat inactivated normal AB human serum and 10 mg/ml of the aforementioned antibodies or 10% pooled HD or COVID-19 patient plasma. Cultures were then incubated at 37°C, 5% CO₂ for 48 h at which time cells were harvested and fixed for 20 min with 4% PFA and then stained. Immunoglobulin (Ig) from COVID-19 patient pooled plasma was depleted by protein A/G agarose resin. Control samples were incubated with agarose resin without coupled protein. C1A-B12 and C1A-H12, two SARS-CoV-2 Spike-targeting human monoclonal antibodies, were produced as previously described⁶³.

2.2.10 qRT-PCR. RNA extracted from HD monocytes (stimulated or not with LPS (100 ng/ml for 16 h), or COVID-19 patient monocytes was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and the cDNA was analyzed by qRT-PCR using

the Sso FastTM EvaGreen® Supermix (BioRad) (30 sec at 95°C, 40 cycles (3 sec at 95°C; 3 sec at 54 °C) for both *ACE2* and *BSG* (CD147)) using a CFX96 Touch Real-Time PCR Detection System (BioRad). Primer sequences for *ACE2*, *BSG* and *ACTB* are given in **2.2.1**.

2.2.11 Statistical Analysis. Statistical analysis was performed using GraphPad Prism V7.0.

Normal distribution of the data was evaluated by the D'Agostino and Pearson normality test prior to applying statistical methods. Distributions were considered normal if $P \leq 0.05$. Parametric or non-parametric (Mann-Whitney or Kolmogorov-Smirnov tests) two-tailed unpaired *t*-tests were used to compare two unpaired groups. Multiple group comparisons were analyzed by one-way ANOVA with Sidak's or Tukey's multiple comparisons tests, or non-parametric Kruskal-Wallis with Dunn's post-test. Multiple groups were compared by two-way ANOVA with additional Sidak's or Tukey's multiple comparisons test. Mean plasma values from hospitalized COVID-19 patients on each day were compared between severity groups by multiple unpaired *t*-tests. Correlations of plasma levels were determined by simple linear regression and Pearson correlation coefficient. Differences were considered statistically significant when $P \leq 0.05$.

2.3 Results

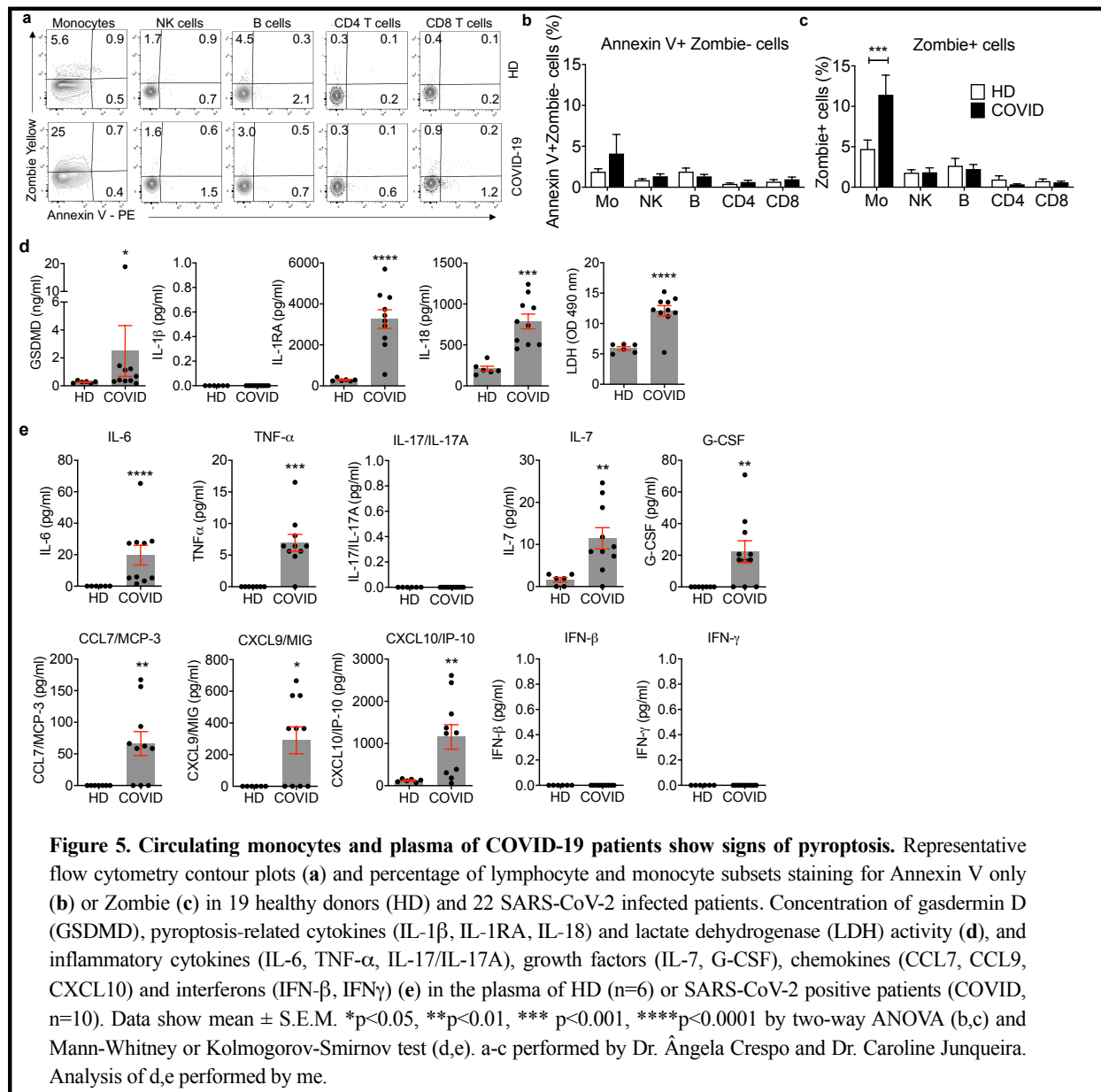
2.3.1 Circulating monocytes and plasma of COVID-19 patients show signs of pyroptosis. To

analyze cell death, freshly isolated mononuclear cells from 19 healthy donors (HD) and 22 COVID-19 patients seen in the emergency department (ED) of Massachusetts General Hospital were stained for hematopoietic cell markers, a small fixable dye (Zombie Yellow) that enters cells whose plasma membrane is damaged, and annexin V, which identifies cells undergoing programmed cell death (Fig. 5a-c, Supplemental Fig. 1a). Although there was no increase in annexin V+ Zombie- apoptotic cells in any subpopulation in COVID-19 samples, on average ~11% of monocytes of COVID-19 patients took up Zombie dye, a sign of ongoing membrane

damage consistent with pyroptosis. Consistent with previous reports⁶⁴, a reduced proportion of CD14⁺ CD16⁻ classical monocytes and more CD14⁺ CD16⁺ intermediate monocytes were observed in COVID-19 patients compared to HD ([Supplemental Fig 1b, c](#)). This intermediate state is a sign of monocyte activation⁶⁴.

Many studies have reported signs of inflammation in the plasma of COVID-19 patients, and some have reported increased plasma biomarkers specifically linked to canonical inflammasome and gasdermin activation, such as IL-1 family cytokines and LDH – which is only released in necrotic forms of cell death, including pyroptosis^{65–67}. Elevated LDH is one of the best correlates of severe COVID-19 disease^{19,21}. However, none have looked at plasma GSDMD, which is selectively released during pyroptosis⁶⁸. Plasma from the HD and COVID-19 patients evaluated above was assessed by multiplex ELISA and LDH activity assay for specific inflammatory markers of pyroptosis (GSDMD, IL-1 β , IL-1RA, IL-18, LDH) ([Fig. 5d](#)) and for other markers of inflammation that are not pyroptosis-specific (inflammatory cytokines IL-6, TNF α , IL-17/17A; growth factors IL-7, G-CSF; chemokines CCL7, CXCL9, CXCL10), and for interferons (IFN β , IFN γ) ([Fig. 5e](#)). All of the specific pyroptosis markers, except IL-1 β , were significantly elevated in COVID-19 patient plasma compared to HD. Plasma IL-1 β was below the level of detection in all samples, which was not surprising since it is rapidly cleared from the blood and usually not detected even in patients with severe disease caused by ongoing pyroptosis, such as those bearing constitutively active *NLRP3* mutations⁵⁷ or with IL-1-mediated systemic juvenile idiopathic arthritis⁶⁹. However, its antagonist IL-1RA, which can be used as a surrogate⁵⁷, was greatly increased in COVID-19 samples. IL-6 and TNF α , which can be increased following IL-1 family cytokine binding to IL-1 receptors, and many of the growth factors and chemokines measured were also highly elevated in COVID-19 plasma compared to HD, as has been

reported¹⁶⁵. In contrast, the interferons were not detected in COVID-19 patient plasma, consistent with previous observations that SARS-CoV-2 infection inhibits the interferon response^{53,70}. Taken together, these results suggest COVID-19 patient blood monocytes undergo pyroptosis and release of pro-inflammatory cytokines.



2.3.2 Plasma markers of pyroptosis correlate with severe COVID-19. To determine if pyroptosis-related biomarkers correlate with COVID-19 disease severity, plasma from 60 COVID-19 patients who presented to the ED were analyzed for GSDMD, LDH, and plasma cytokines at presentation and on days 3 and 7 for those patients who were hospitalized (Fig. 6a,b). Patients were grouped into mild, moderate, or severe disease using the MGH COVID Acuity scale^{60,61}. Moderate disease was defined as requiring supplemental O₂ during hospitalization and severe disease was defined as requiring mechanical ventilation and/or causing death. Plasma levels of the pyroptosis-specific markers GSDMD, LDH, IL-1RA, and IL-18 were all elevated in severe patient samples compared to those with mild or moderate disease, but the increase in GSDMD did not reach significance (Fig. 6a). Other markers of inflammation that are not specific for pyroptosis (C-reactive protein, D-dimer, IL-17, CCL7, CXCL10) were significantly increased early during hospitalization in patients who developed severe COVID-19, but others (IL-6, TNF α , CXCL9) were not (Fig. 6b). Although GSDMD levels did not significantly correlate with disease severity, GSDMD plasma levels measured in the ED at presentation correlated significantly with a marker of pyroptosis (IL-1RA) and with some other markers of inflammation (IL-6, G-CSF, CXCL10) (Fig. 6c). Furthermore, plasma IL-1RA also significantly correlated with plasma IL-6, TNF α , G-CSF, and CXCL10 on day 0 (Fig. 6d). Thus, pyroptosis biomarkers at presentation correlate with COVID-19 disease severity and more general markers of inflammation.

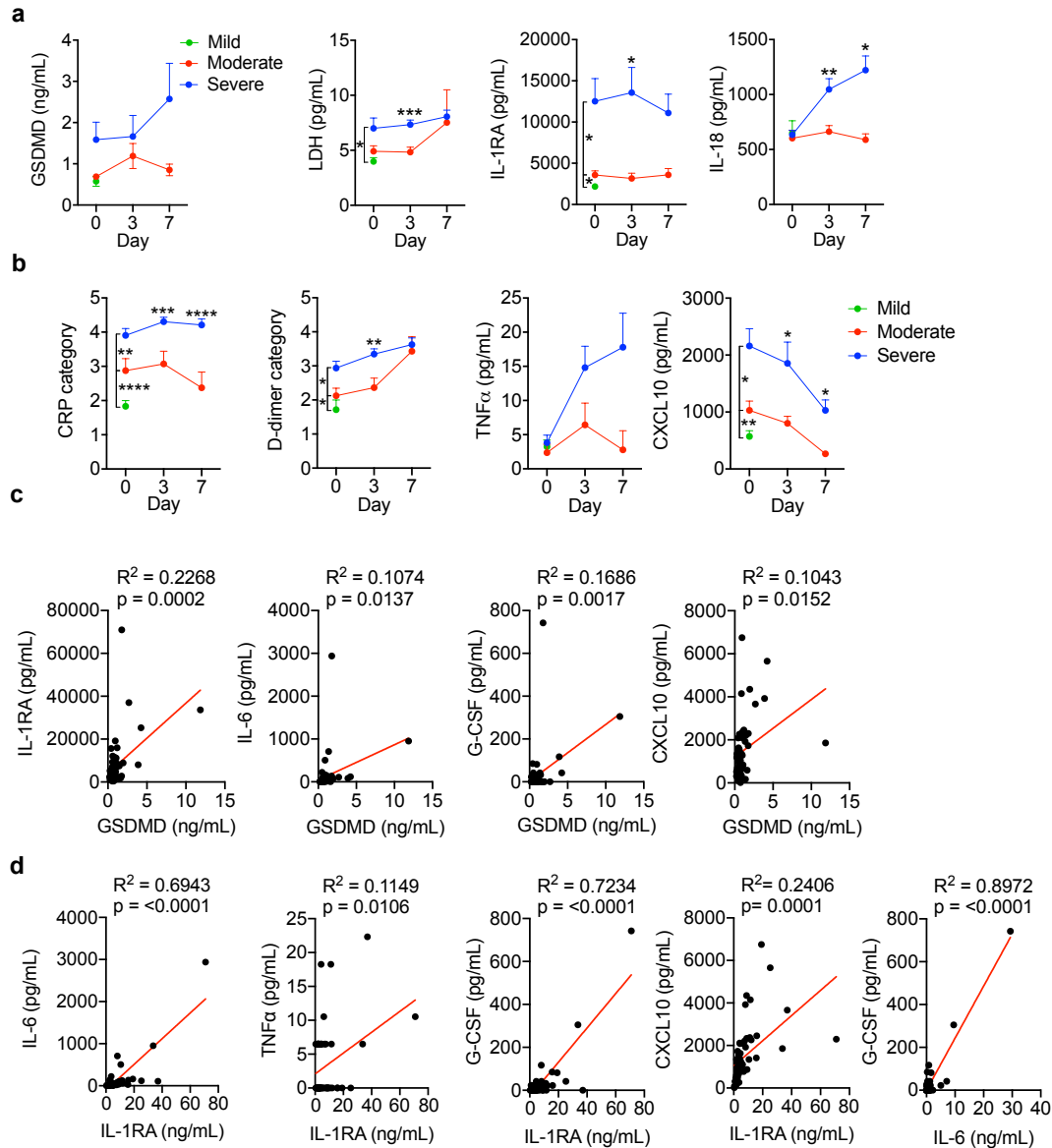


Figure 6. Plasma markers of pyroptosis correlate with severe COVID-19. Plasma pyroptosis biomarkers GSDMD, LDH activity, IL-1RA and IL-18 (**a**), C-reactive protein (CRP), D-dimer, TNF- α and CXCL10 (**b**) in mild, moderate and severe COVID-19 patients (n=60) over time during hospitalization. CRP and D-dimer values were grouped as described in the methods. **c**, Correlation of GSDMD levels in the plasma of COVID-19 patients (at time of diagnosis, day 0) with IL-1RA, IL-6, G-CSF, and CXCL10. **d**, Correlation of day 0 plasma IL-1RA with IL-6, TNF α , G-CSF and CXCL10, and of IL-6 with G-CSF. Data shown are mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (by multiple t-tests (**a,b**) and simple linear regression and Pearson correlation (**c,d**)). Analysis of a-d performed by me.

2.3.3 Circulating monocytes have activated NLRP3 and AIM2 inflammasomes. The above data suggested that circulating monocytes in COVID-19 patients might die of pyroptosis and release inflammatory cytokines that could contribute to poor clinical outcomes. There are 27 potential human canonical inflammasome sensors (22 NOD-like receptors (NLR), 4 AIM2-like receptors (ALR) and pyrin), but not much is known about how viruses interact with them²². The NLRP3 inflammasome, which detects K⁺ efflux generated by a variety of stimuli including extracellular ATP, bacterial toxins, or disruption of the cell membrane, could be activated by lytic SARS-CoV-2 infection itself or by specific viral proteins^{71,72}. Three SARS-CoV-2 proteins, Orf3a, Orf8b, and the E envelope protein, are “viroporins” (ion channels) that activate NLRP3 by K⁺ efflux when ectopically expressed^{37,42,73,74}. Orf3 and Orf8 are encoded by the pathogenic, but not the avirulent, human coronaviruses³⁷, suggesting that NLRP3 activation might differentiate virulent from avirulent coronavirus infection. Interestingly, bats, the natural zoonotic hosts of SARS-CoV and SARS-CoV-2, have a dampened NLRP3 response to infection with multiple viruses, including MERS-CoV, which might explain their ability to tolerate these infections despite high viral loads^{46,47}.

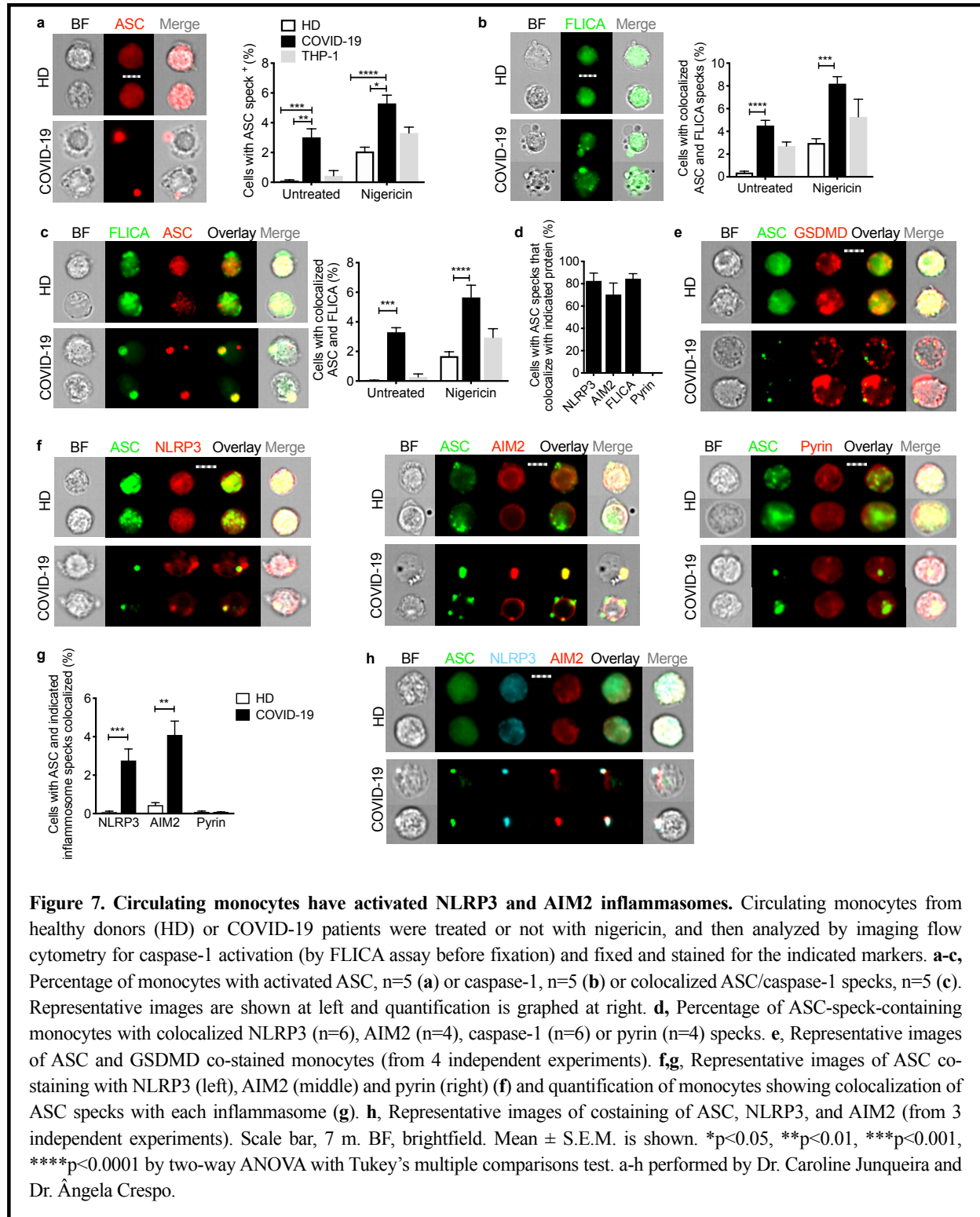
To probe whether circulating monocytes from COVID-19 patients are undergoing pyroptosis, freshly isolated, purified monocytes from 3-8 HD or COVID-19 patients were stained for the common inflammasome adapter ASC, activated caspase-1 (FLICA), and GSDMD and analyzed by imaging flow cytometry for their expression and intracellular distribution ([Fig. 7 and Supplemental Fig. 2](#)). When a canonical inflammasome is activated, a large micron-sized inflammasome-ASC-caspase-1 speck is formed²². A significant proportion of freshly isolated monocytes from COVID-19 patients, compared to HD, showed activated caspase-1 and ASC specks ([Fig. 7a-c](#)), confirming that circulating monocytes in COVID-19 patients are

spontaneously undergoing pyroptosis. Most of the cells with ASC specks (>80%) also had co-localized activated caspase-1 specks, visualized by fluorochrome-labeled inhibitor of caspases (FLICA) assay (Fig. 7d). When these monocytes were treated with a low concentration of nigericin (20 μ M) for 30 minutes, which triggers the NLRP3 inflammasome, all the samples had some cells with ASC-caspase-1 specks, but in the COVID-19 samples these were increased approximately three-fold, indicating that they were more prone to pyroptosis. As a control, a monocytic cancer cell line (THP-1) was treated with nigericin.

As expected, GSDMD did not co-localize with ASC-caspase-1 specks in any of the samples (Fig. 7e). GSDMD staining was different in ASC speck+ COVID-19 patient monocytes compared to HD and to ASC speck-negative COVID-19 patient monocytes – in cells with ASC specks, GSDMD redistributed from mostly cytoplasmic staining to form prominent cell membrane puncta, consistent with GSDMD cleavage and pore formation (Fig. 7e and Supplemental Fig. 2b).

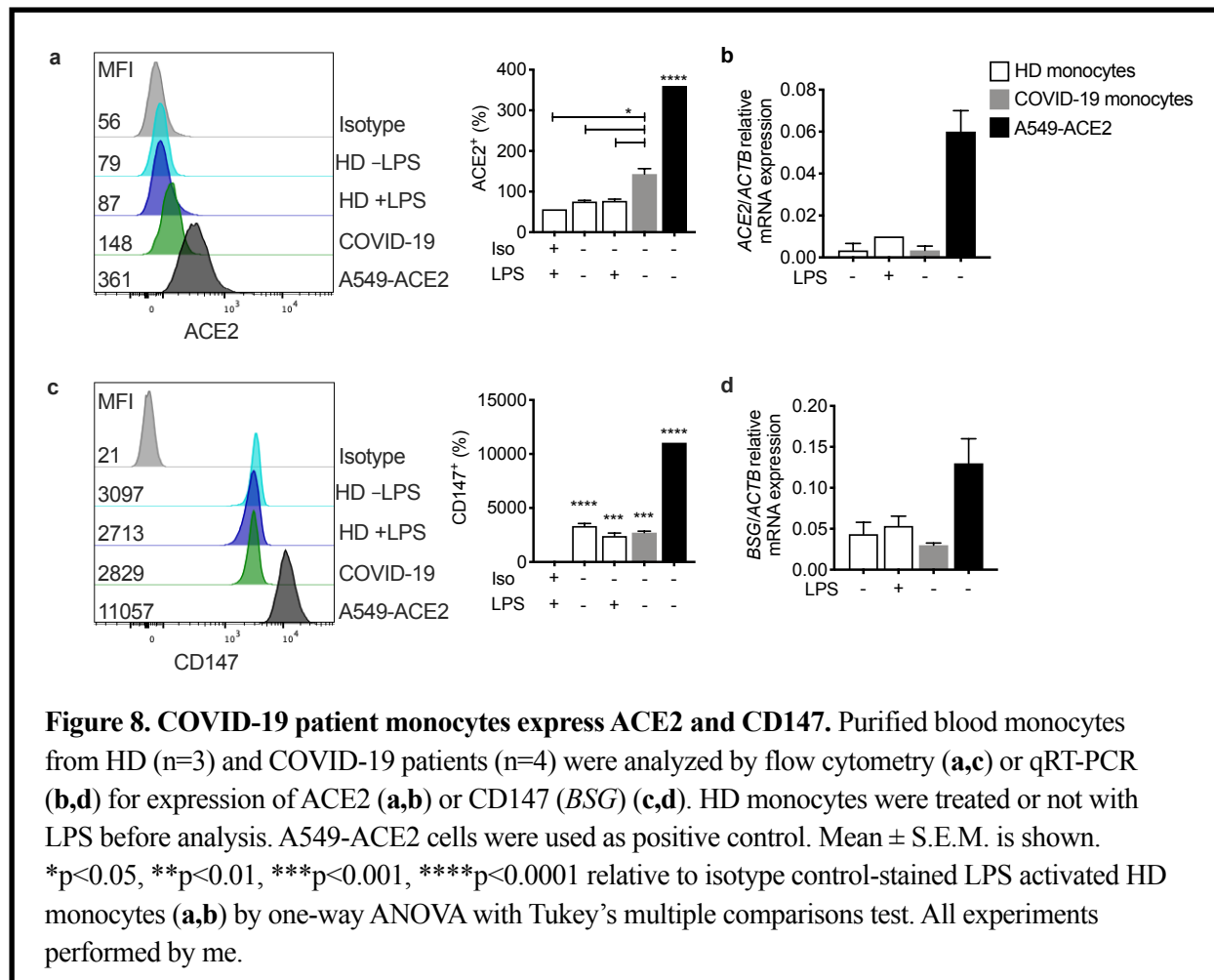
To identify the inflammasome sensor activated in COVID-19 patient monocytes, freshly isolated HD and COVID-19 patient monocytes were co-stained for ASC and 3 canonical inflammasomes (NLRP3, AIM2 (activated by cytoplasmic DNA) or pyrin (activated by bacterial toxins, as a negative control)) (Fig. 7d, f-h)^{75,76}. COVID-19 patient monocytes with ASC specks co-localized NLRP3 and AIM2 with ASC specks, but there were no pyrin specks, as expected. Thus, both NLRP3 and AIM2 were activated to form inflammasomes in COVID-19 patient monocytes. The activation of the DNA sensor AIM2 was unexpected, although AIM2 has been shown, in rare cases, to be activated by RNA viruses by an unclear mechanism^{77–79}. AIM2 might also sense host genomic or mitochondrial DNA – notably, mitochondrial membranes are reported to be damaged during pyroptosis⁸⁰. Because assessment of cellular co-localization is more convincing using

confocal microscopy than by imaging flow cytometry, Z stacks and 2D projection of confocal microscopy images of HD and COVID-19 patient monocytes co-stained for ASC and activated caspase-1, NLRP3, AIM2, or pyrin inflammasomes were also analyzed. Confocal images confirmed the imaging flow cytometry co-localizations ([Supplemental Fig. 2c](#)). Almost all of the cells with ASC specks had co-localized NLRP3 and AIM2 specks ([Fig. 7d](#)), and ASC, NLRP3, and AIM2 co-localized together ([Fig. 7h](#)). These data demonstrate that NLRP3- and AIM2-ASC-caspase-1 inflammasomes are activated and GSDMD relocates to the cell membrane in a significant fraction of circulating monocytes in COVID-19 patients. Taken together with the large proportion of dying Zombie⁺ annexin V- circulating monocytes we observed ([Fig. 5a-c](#)), these data indicate that some blood monocytes are dying of pyroptosis in COVID-19 infected patients.



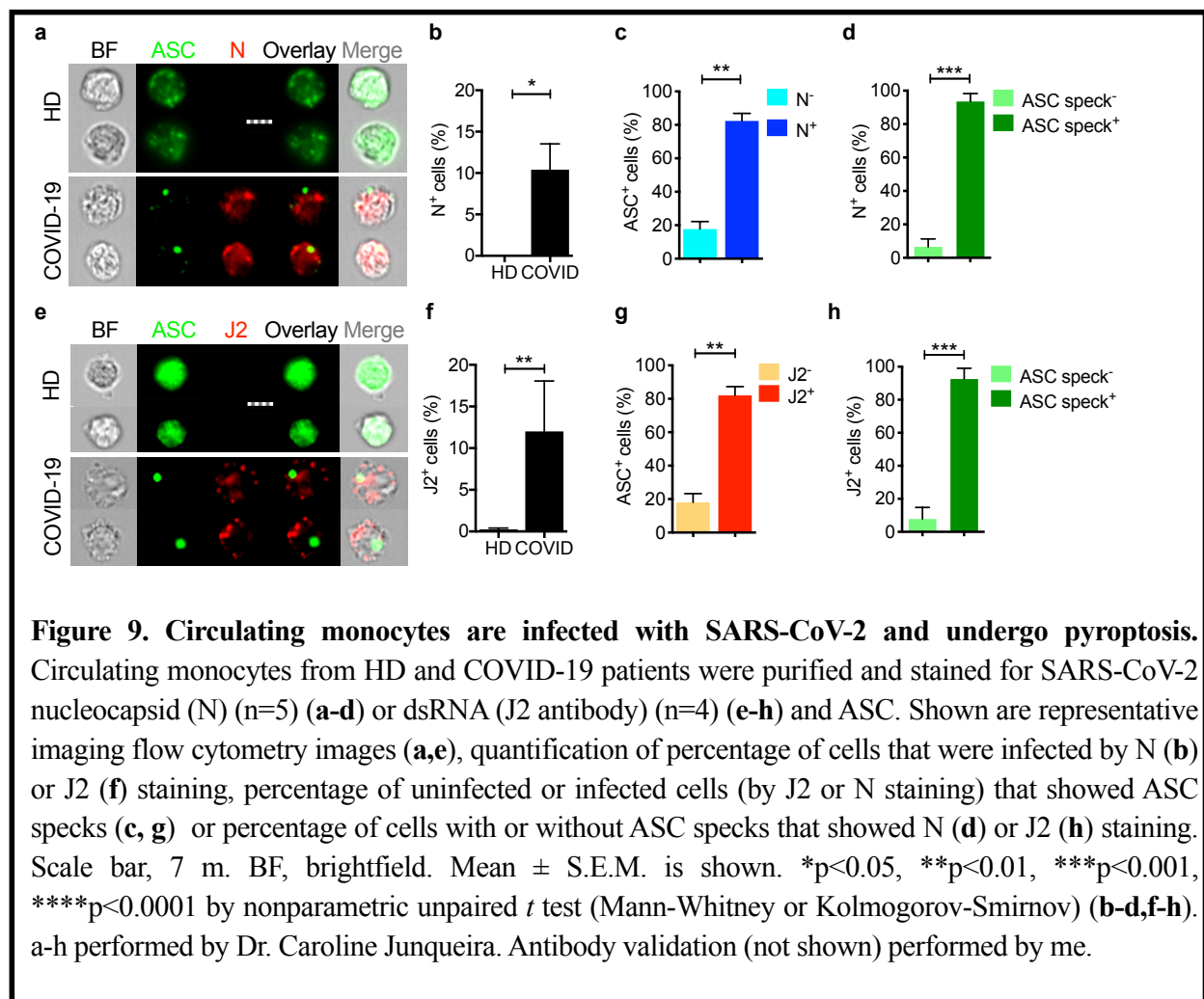
2.3.4 Circulating monocytes are infected with SARS-CoV-2 and undergo pyroptosis. The observation that some COVID-19 patient monocytes undergo pyroptosis suggested that

circulating monocytes might take up SARS-CoV-2, which may in turn activate the NLRP3 and AIM2 inflammasomes and pyroptosis. Monocytes are not thought to express the receptor ACE-2, which serves as the dominant entry receptor for SARS-CoV-2, but this finding has been disputed^{81,82}. Although ACE-2 expression was not detected in HD monocytes, even after LPS activation, COVID-19 patient monocytes appeared to dimly express ACE-2 by flow cytometry and qRT-PCR (Fig. 8a,b). Both HD and COVID-19 patient monocytes also expressed similar levels of CD147 (basigin or EMMPRIN), an immunoglobulin superfamily receptor implicated in bacterial, parasite, and viral entry, which has been reported to bind to SARS-CoV-2 spike protein and facilitate viral uptake and infection⁸³. Similar to ACE-2, CD147 expression in HD monocytes did not change after LPS stimulation (Fig. 8c,d). Monocytes also express 2 Fc gamma receptors – CD32 (FcγRII, expressed on most blood monocytes⁸⁴) and CD16 (FcγRIII, expressed on a small minority of blood monocytes that are activated and increased in number in COVID-19 patients)⁶⁴ (Supplemental Fig. 1c). These Fcγ receptors could recognize antibody-opsonized viral particles and mediate their entry into blood monocytes via antibody-dependent phagocytosis (ADP)⁸⁵.



Anti-SARS-CoV-2 spike protein antibodies are detected at about the same time that patients start developing inflammatory symptoms^{86–89}. Two recent reports suggest that monocytes might be infected with SARS-CoV-2^{67,90}. To examine whether COVID-19 patient blood monocytes can be infected and support SARS-CoV-2 replication and to determine whether infection triggers inflammasome activation, we co-stained isolated HD and COVID-19 patient monocytes for SARS-CoV-2 nucleocapsid (N) (Fig 9a-d) or dsRNA (J2 antibody) (Fig 9e-h) and ASC. While cells that passively take up virions or contain replicating virus would be expected to stain for N, J2 staining generally indicates active viral replication⁹¹. Although HD monocytes did not stain for N, dsRNA or ASC specks, ~10% of the blood monocytes from SARS-CoV-2

infected patients on average stained for N or dsRNA (Fig 9b,f), suggesting that a minority of circulating monocytes in COVID-19 patients contain actively replicating SARS-CoV-2. Moreover, virtually all of the infected cells showed ASC specks (Fig 9c,g) and virtually all of the cells with ASC specks were infected (Fig 9d,h). Thus, SARS-CoV-2 infection of blood monocytes is associated with inflammasome activation and pyroptosis.



2.3.5 Healthy donor monocytes are inefficiently infected with SARS-CoV-2, but infection is enhanced by anti-Spike antibody, patient plasma, or stimulation with LPS. To examine the susceptibility of monocytes to SARS-CoV-2 and begin to elucidate how they might become

infected, purified HD monocytes were infected with an engineered infectious clone (icSARS-CoV-2-mNG) derived from the 2019-nCoV/USA_WA1/2020 strain and encoding a Neon Green (NG) fluorescent protein gene as a reporter of viral replication⁶². Monocytes, primed or not with LPS, were infected (MOI 1) with the reporter virus that was preincubated with IgG₁ isotype control antibody (mAb114), anti-Spike mAbs (non-neutralizing C1A-H12, neutralizing C1A-B12)⁶³ or pooled HD or COVID-19 patient plasma (heat inactivated or not). Antibodies and plasma were also present during the 48 h culture. After 48 h, monocytes were co-stained for dsRNA (J2 antibody) or SARS-CoV-2 N and ASC and analyzed by imaging flow cytometry (Fig. 10, Supplemental Fig. 3). Staining for N indicates virus internalization, whereas J2 staining and NG fluorescence indicate viral replication. Without LPS or anti-Spike antibody or COVID-19 pooled plasma, very few, if any, HD monocytes took up or replicated the virus, but virus uptake and replication increased significantly in the presence of anti-Spike mAb or COVID-19 plasma. The neutralizing activity of the antibody did not appear to strongly affect infection. HD plasma did not augment HD monocyte infection and heat inactivation of COVID-19 plasma did not significantly alter its ability to potentiate infection. These findings suggested that complement did not facilitate monocyte infection and that specific antiviral antibodies that opsonize viral particles might be involved (Supplemental Fig. 3). Immunoglobulin (Ig)-depletion of COVID-19 plasma nearly abrogated viral infection assessed by J2 staining and NG fluorescence (Fig. 10k,l). Thus, viral entry in monocytes occurs predominantly by ADP of opsonized viral particles, but further work is needed to probe which Fc receptors are involved. The highest infection rate was ~3% in HD monocytes pretreated with LPS and incubated with patient plasma. N and J2 staining were comparable across the different conditions with a low background of ~0.1% in uninfected samples; fewer cells were NG fluorescent and there was no

background NG fluorescence in uninfected samples. More of the J2 or N staining cells in the samples with the highest infection rates (treated with LPS and patient plasma or anti-Spike antibodies) were also NG fluorescent, (Fig. 10i,j, Supplemental Fig. 3g,h). These results seem to indicate that the virus replicates in most monocytes that take it up, but NG expression is detected only in cells with more vigorous replication. In particular, LPS activation enhances SARS-CoV-2 infection and viral replication in monocytes. LPS pretreatment also slightly increased the frequency of J2 and N staining when anti-Spike or patient plasma were not added, suggesting that another non-Fc receptor on activated monocytes, such as ACE-2 or CD147, might weakly mediate viral uptake. Further work will be needed to identify how monocyte activation enhances monocyte infection in SARS-CoV-2 infection.

As in monocytes from COVID-19 patients, *in vitro* SARS-CoV-2 infection of HD monocytes led to ASC speck formation (Fig. 10a,b, f-h, Supplemental Fig. 3). The numbers of infected HD monocytes with ASC specks under the various conditions (with and without antiviral antibodies or plasma, and with or without LPS) roughly paralleled and were comparable to the numbers of anti-N or J2 staining cells. Furthermore, virtually all of the N or J2 positive cells had ASC specks, suggesting that once a monocyte is infected with SARS-CoV-2, whether in COVID-19 patients *in vivo* or in *ex vivo* infection of HD monocytes, SARS-CoV-2 infection stimulates the formation of an inflammasome and pyroptosis.

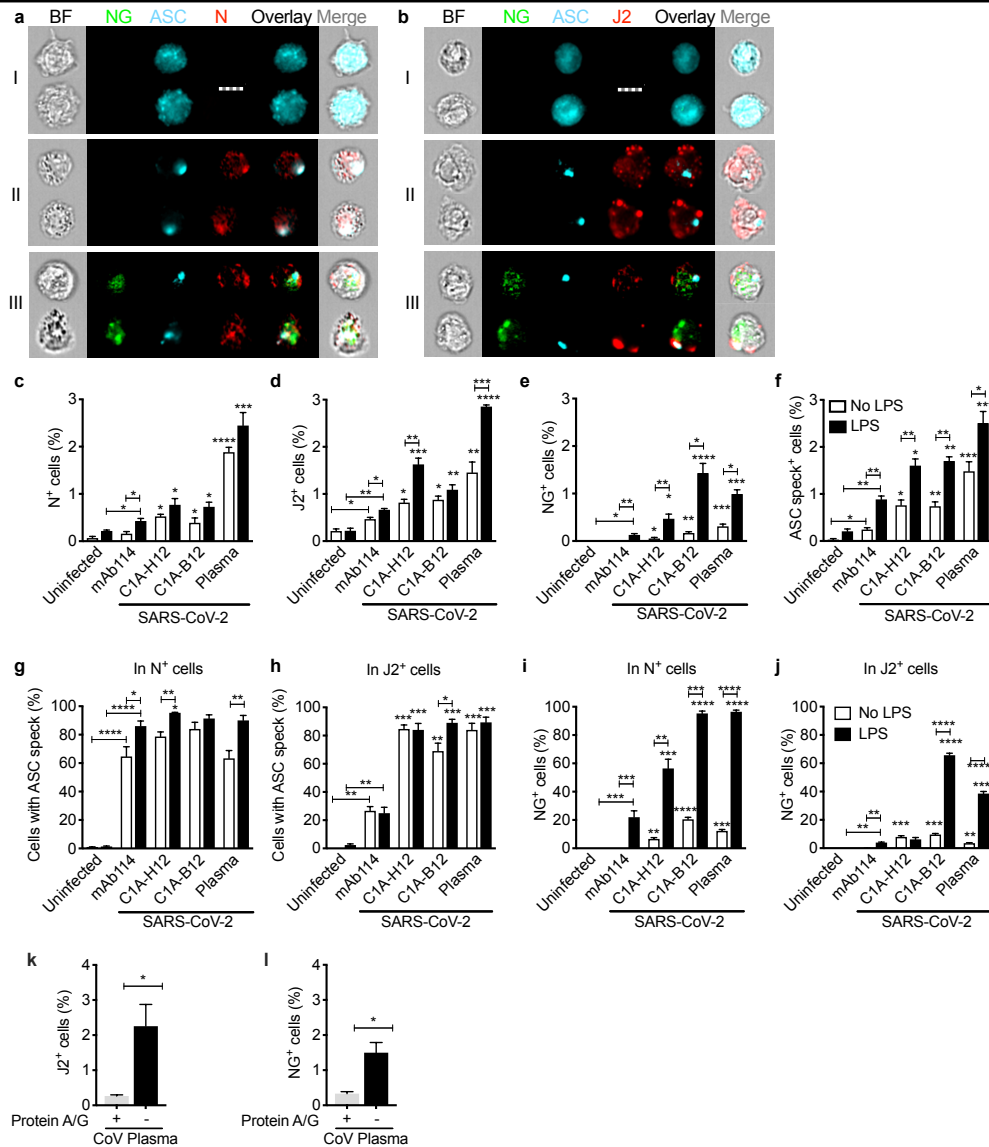


Figure 10. *In vitro* infection of healthy donor monocytes. Healthy donor (HD, n=3) monocytes were primed or not with LPS, infected with icSARS-CoV-2-mNG (MOI, 1), then stained 48 h later for nucleocapsid (N) or dsRNA (J2) and ASC. Before infection, virus was preincubated with indicated monoclonal antibodies (IgG1 isotype control mAb114), non-neutralizing anti-spike (C1A-H12) or neutralizing anti-RBD (C1A-B12) or with pooled COVID-19 patient plasma (Plasma). Antibodies or plasma were present throughout the culture. **a,b**, Representative imaging flow cytometry images of (I) uninfected monocytes, (II) N or J2 staining cells without detectable Neon green (NG), or (III) with NG and N or J2 staining. **c-f**, Quantification of HD monocytes staining for N (**c**), J2 (**d**), NG (**e**) or ASC specks (**f**). **g,h**, Percentage of N⁺ (**g**) and J2⁺ (**h**) cells that had ASC specks. **i,j**, Percentage of N⁺ (**i**) and J2⁺ (**j**) cells that had detectable NG. **k,l**, Percentage of J2⁺ (**k**) and NG fluorescent (**l**) LPS-activated monocytes after infection with icSARS-CoV-2-mNG virus that was preincubated with CoV pooled plasma that was pretreated with Protein A/G-coated agarose beads or with uncoated agarose beads to deplete or not immunoglobulins. BF, Brightfield. Scale bar, 7 μ m. Mean $\pm$ S.E.M. is shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ relative to Iso or as indicated by two-way ANOVA with Sidak's multiple comparisons test and unpaired non-parametric t test (**k,l**). Infection performed by Dr. Shahin Ranjbar, analysis performed by Dr. Caroline Junqueira. Antibody validation (not shown) performed by me.

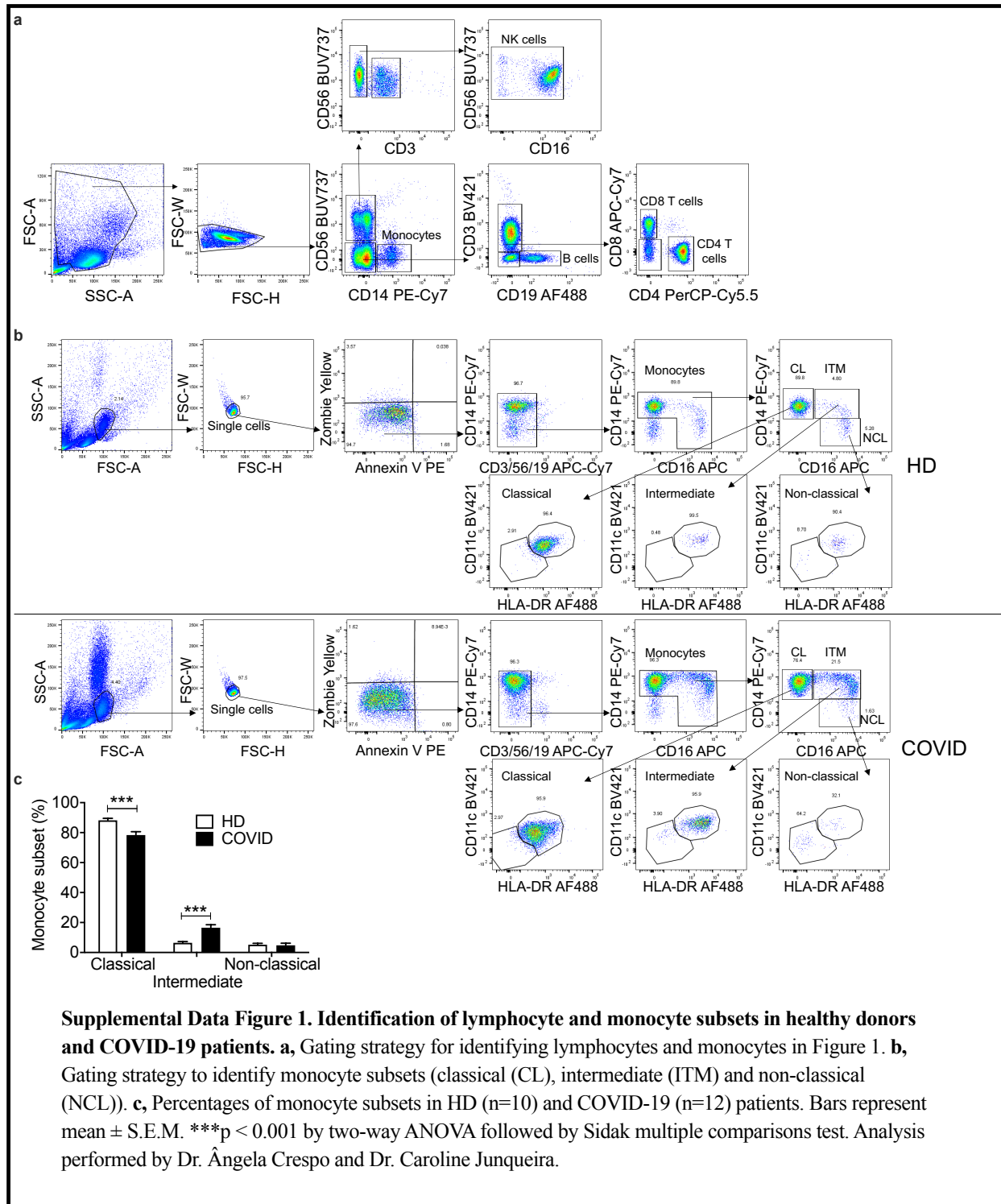
2.4 Conclusion

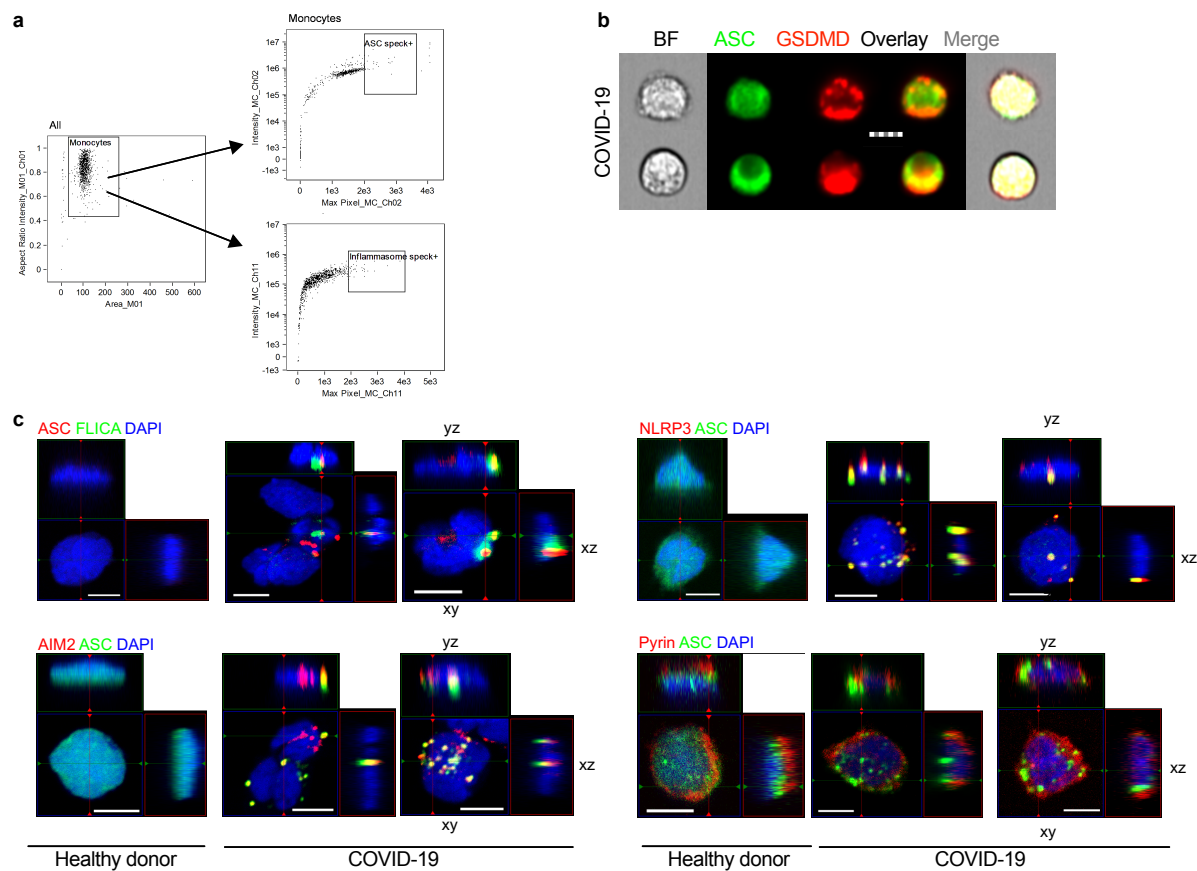
Myeloid antigen-presenting cells such as monocytes, macrophages, and dendritic cells are sentinels that kickstart the innate immune response by sensing infection and danger, which activate inflammasomes. These myeloid cells are important sources of potent inflammatory IL-1 family cytokines, whose processing and release depends on canonical inflammasome activation of caspase-1 and the pore-forming protein GSDMD²². My co-workers and I found that antibody-opsonized SARS-CoV-2 can infect and replicate in blood monocytes, which have activated inflammasomes and undergo pyroptosis. There is a one-to-one correspondence between monocyte infection and inflammasome-caspase-1 activation. Infected blood monocytes from COVID-19 patients have activated NLRP3-ASC-caspase-1 and AIM2-ASC-caspase-1 inflammasomes. It is unclear how these inflammasomes are activated, but NLRP3 senses cell membrane damage, which may be caused by one or more of three putative viroporins – ORF3a, ORF8, and E – expressed by SARS-CoV-2. It is surprising that an RNA virus such as SARS-CoV-2 activates the DNA sensor AIM2, and further work must be done to determine if the DNA comes from the host or the virus, but it is possible that mitochondrial DNA activates AIM2 following mitochondrial damage that can occur during pyroptosis.

Plasma biomarkers of pyroptosis including IL-1RA, IL-18, and LDH correlate with development of severe disease in this patient cohort, indicating they may be useful as part of a diagnostic panel to predict who may be susceptible to an overly exuberant inflammatory response and severe disease. These results also suggest that immune modulating therapy may be helpful in preventing severe COVID-19. Although IL-1 β and IL-6 antagonism have had mixed results in clinical trials^{26–29}, two FDA-approved inhibitors of GSDMD, the pore-forming protein responsible for pyroptosis and IL-1 family cytokine release – disulfiram (Antabuse)³⁰ and

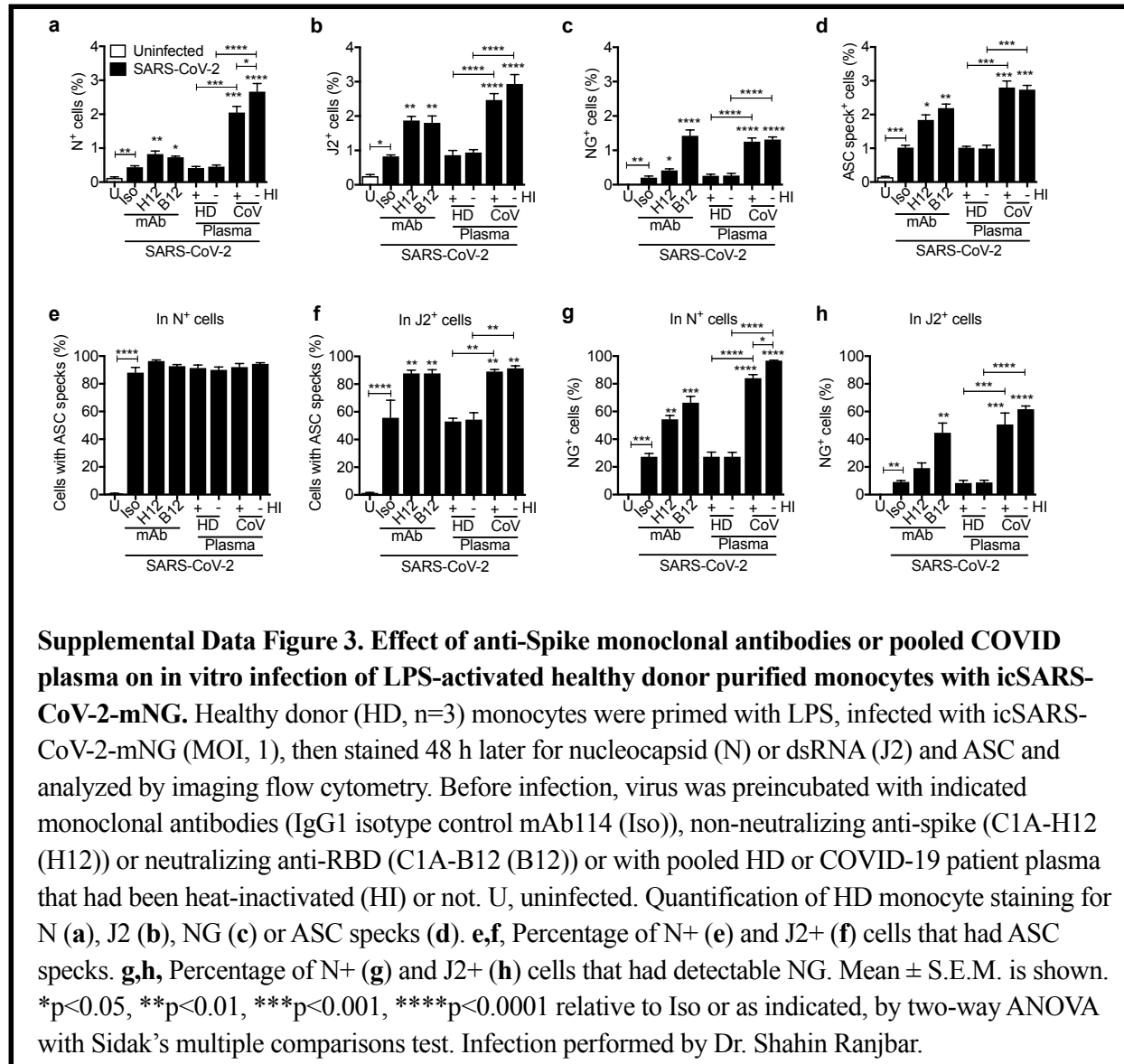
dimethyl fumarate (Tecfidera)³¹ – are worth evaluating. Taken together, the above results suggest blood monocyte infection, inflammasome activation, and cytokine processing and release play a role in the pathology of CRS in severe COVID-19.

2.5 Supplemental Data





Supplemental Data Figure 2. Representative images of inflammasome speck formation in COVID-19 patient monocytes. **a**, Gating strategy for imaging flow cytometry analysis of isolated monocytes. **b**, Representative imaging flow cytometry images of GSDMD and ASC co-staining in COVID-19 patient monocytes that lacked ASC specks. Scale bar, 7 m. **c**, Representative confocal image z-stacks and plane projections of monocytes of HD and COVID-19 patient monocytes, stained for the same markers as in Figure 7. Scale bars, 5 μm. Microscopy performed by Dr. Ângela Crespo.



Chapter 3: Discussion

3.1 Overview

During infection, myeloid cells produce large amounts of inflammatory cytokines that alert the innate immune system to infection and danger. IL-1 family cytokines are important inflammatory mediators produced by myeloid cells, but their processing and release depend on canonical inflammasome activation of caspase-1 and GSDMD^{22,92}. In this thesis, my colleagues and I show that inflammatory cytokines, including IL-1 β and IL-18, growth factors, and markers of pyroptosis correlate with development of severe COVID-19. Additionally, up to 10% of COVID-19 patient blood monocytes can be infected by SARS-CoV-2, and a comparable number took up a membrane-impermeable dye, indicating pyroptosis. There was a one-to-one correspondence between infection and NLRP3 and AIM2 inflammasome activation, and these two inflammasomes almost always colocalized with each other, ASC, and active caspase-1. Purified monocytes from healthy donors can be infected by SARS-CoV-2 *ex vivo*, though infection is enhanced by monocyte activation with LPS, and depends on anti-Spike antibodies or patient plasma. Importantly, infection was abrogated by IgG depletion of the plasma, indicating a role for antibody-mediated uptake of opsonized viral particles in monocyte infection. Together, these results suggest that antibody-mediated uptake of SARS-CoV-2 facilitates productive infection of blood monocytes, leading to NLRP3 and AIM2 inflammasome activation of caspase-1 and GSDMD, IL-1 family cytokine processing and release, and pyroptosis. The release of cytokines and other inflammatory mediators during pyroptosis likely contributes to CRS that characterizes severe COVID-19, raising the possibility that blocking pyroptosis may have therapeutic benefit to prevent severe COVID-19.

3.2 Significance of Findings

While several studies have shown increased IL-1 family cytokines in COVID-19 patient plasma, *in vitro* infection of myeloid cells by SARS-CoV-2, or NLRP3 activation in COVID-19 patient blood cells, none of these studies have shown that monocyte infection is antibody-mediated and leads to replication, nor have they tied monocyte infection to inflammasome activation and pyroptosis^{65–67,90}. Our results implicate antibody-mediated monocyte infection, inflammasome activation, and pyroptosis in the development of severe COVID-19. The implication of a critical role for antibodies in facilitating infection of monocytes that do not express the spike cell surface receptor ACE2 raises the question of possible antibody-dependent enhancement of SARS-CoV-2 infection, and whether antibodies produced following vaccination for COVID-19 or passively administered to treat severe disease may also enhance infection. While there is currently no evidence for enhanced infection following vaccination, it will be important to determine if vaccination-induced antibodies have the capacity to mediate infection of monocytes or other myeloid cells.

Our results also raise the possibility of new therapeutic approaches to prevent severe COVID-19 by inhibiting inflammasome activation or pyroptosis. Since pyroptosis and IL-1 family cytokine release depend on the pore-forming protein GSDMD, GSDMD activation is a possible bottleneck for CRS and severe disease. Two FDA-approved drugs – disulfiram (Antabuse)³⁰ and dimethyl fumarate (Tecfidera)³¹ – block GSDMD-mediated pore formation and have been shown to reduce plasma levels of inflammatory cytokines and improve clinical outcomes in a mouse model of sepsis. Since pyroptosis has been linked to CRS in sepsis and sepsis pathology overlaps considerably with severe COVID-19 symptomatology, similar

therapeutic interventions may be useful in preventing CRS and severe disease during SARS-CoV-2 infection.

3.3 Remaining Questions

There are still several important questions that must be investigated to further understand the mechanisms underlying monocyte infection and inflammasome activation. While we found that monocyte infection depends on antibody-mediated uptake of SARS-CoV-2, what receptor mediates internalization remains to be elucidated. The most likely candidates are CD16 (FcγRIIIa) and CD32 (FcγRIIa), which are both expressed on innate immune cells including monocytes, neutrophils, eosinophils, and macrophages⁸⁵. Both these receptors are activating receptors that bind to IgG Fc regions and signal through immunoreceptor tyrosine activating motifs (ITAMs) to induce inflammatory cytokine production, such as TNFα and IL-8. When Fcγ receptors are cross-linked, as can happen when binding to opsonized virions, the antibody-virion complexes are phagocytosed and virions are degraded by the acidic environment and enzymes within the phagolysosome⁸⁵. However, in some infections, such as Dengue virus, acidification of the phagolysosome can promote viral fusion and infection, thus resulting in antibody-dependent enhancement (ADE)⁸⁵. It is unknown if this occurs during SARS-CoV-2 infection, but our studies show blocking CD16 abrogates infection of healthy donor monocytes (not shown), so it is possible a similar mechanism of ADE occurs with SARS-CoV-2.

A recent study implicated CD32b (FcγRIIb) binding to neutralizing antibodies from COVID-19 patient plasma in dampening the IFN response to SARS-CoV-2 infection⁹³. CD32b is an inhibitory receptor with similar expression profiles as CD32a and CD16. CD32b acts as a counterbalance to activating Fcγ receptors by signaling through an immunoreceptor tyrosine inhibitory motif (ITIM) that recruits phosphatases that inhibit the recruitment and activation of

downstream signaling effectors such as PLC γ and BTK⁸⁵. Thus, the balance between activating and inhibiting Fc γ receptor signaling determines if phagocytes will be activated, and has been implicated in determining the maturation state of dendritic cells⁸⁵. It will be interesting to see if blocking CD32a or CD32b prevents *ex vivo* infection of healthy donor monocytes.

We observed viral replication within infected monocytes. Infected COVID-19 patient monocytes stained for nucleocapsid and J2 (dsRNA) by flow cytometry. While nucleocapsid does not indicate active replication since it may be found in monocytes that took up virus without replication, J2 stains for dsRNA, an intermediate of viral replication that should not be detected if replication does not occur. For our *ex vivo* infection of healthy donor monocytes, we used a clone of SARS-CoV-2 that expresses Neon Green as a reporter for replication⁶² and found that Neon Green is expressed in infected monocytes, indicating active viral replication. To further confirm that SARS-CoV-2 replicates within infected monocytes, droplet digital PCR can be used to detect viral sub-genomic RNA, which is produced during viral replication. Sub-genomic RNAs encode the conserved structural proteins and accessory proteins and are produced by the Nsp12-encoded RNA-dependent RNA polymerase following translation of the viral genomic RNA into nonstructural proteins during replication⁴⁸. Plaque assay using supernatant from the *ex vivo* infection of healthy donor monocytes will also show whether replicating virus is present in monocytes following infection.

However, it is unknown what determines if the virus will replicate. Viral replication seemed to be more vigorous in LPS-activated healthy donor monocytes, suggesting that monocyte activation may amplify viral replication. Other activating signals are worth investigating for their ability to promote productive viral infection. Prostaglandin E₂ (PGE₂) can amplify inflammatory cytokine production by activating NF- κ B, the master transcriptional

regulator of pro-inflammatory cytokines and chemokines, including IL-1 β and IL-18^{94,95}. Plasma PGE₂ is increased in COVID-19 patients, and PGE₂ has also been shown to inhibit type I IFN production and the ISG response, as is seen in severe COVID-19, making it an attractive candidate for monocyte activation during infection⁹⁶. The effects of IFN γ and TNF α on viral replication are also worth investigating since both of these cytokines can prime macrophages through NF- κ B, an important step for the production of pro-inflammatory cytokines and inflammasome components prior to inflammasome activation⁹⁷⁻⁹⁹. It will also be worth activating toll-like receptors 1, 2, 4, 5 and 6, which signal through NF- κ B to promote inflammatory cytokine production. We have already shown the effect of TLR4 activation with LPS on viral infection in monocytes, but TLR1, 2, 5 and 6 can be activated by bacterial tri-acyl lipopeptide, peptidoglycan, flagellin and zymosan, respectively¹⁰⁰. Ultimately, the goal of all these approaches is to prime monocytes by activating NF- κ B prior to infection since priming of monocytes with LPS significantly increased infection in our studies.

The effect of these activating signals on viral infection and replication should be confirmed by further studies utilizing a virus clone with a reporter for replication, such as the Neon Green-expressing clone⁶², as well as by measuring replication by sub-genomic RNA or J2 staining, which are indicators of active viral replication, not just virion uptake. One limitation in such a study is that viral uptake is increased by monocyte activation, so it may be difficult to differentiate if increased levels of replication in activated monocytes are due to more vigorous replication or just increased uptake.

In addition to monocytes, other ACE2- and TMPRSS-expressing lung epithelial cells, such as pneumocytes, are infected and may undergo pyroptosis. The ability of these cell types to undergo pyroptosis depends on their expression of inflammasome components, which varies

between tissues. NLRP3, AIM2, GSDMD, caspase-1, and IL-1 β are expressed in lung epithelium, so they might undergo pyroptosis following SARS-CoV-2 infection^{79,101,102}. Tissue myeloid cells such as macrophages and dendritic cells, especially in the lungs, may also be infected and undergo pyroptosis, but this has yet to be shown. One way to do this would be to stain lung slices for macrophage and dendritic cell markers along with nucleocapsid or spike, NLRP3, AIM2, ASC, GSDMD, or active caspase-1 and look for inflammasome speck formation within these cell types by microscopy.

Another question for further exploration is what other inflammasomes are activated during SARS-CoV-2 infection, both in blood monocytes and in other tissue-resident infected cells. While we found that NLRP3 and AIM2 are activated while Pyrin is not, there are 27 potential human inflammasome sensors, and what most sense is unknown²². Other possible sensors of SARS-CoV-2 infection include CARD8 and NLRP1, which are activated by pathogen proteases during human immunodeficiency virus, human rhinovirus, and *Bacillus anthracis* infection^{103–105}. NLRP9b senses RNA virus infection, but its expression outside intestinal epithelial cells is unknown¹⁰⁶. The RNA helicase RIG-I also senses RNA virus infection, and can activate caspase-1 by directly binding ASC or by activating the CARD9 adaptor protein¹⁰⁷.

Similarly, one unanswered question raised by this thesis is how NLRP3 and AIM2 are being activated. Are they activated directly by SARS-CoV-2, or indirectly? It is possible that NLRP3 is activated by K⁺ efflux caused by one of several putative viroporins encoded by SARS-CoV-2 – ORFs 3a, 8, or E^{43,108}. However, most studies implicating these viroporins in inflammasome activation were done using the homologous, but not identical, ORFs from SARS-CoV, and all studies relied on overexpression of the ORF, and often also of the inflammasome components^{20,37,40–45,73,74}. While the available evidence suggests ORFs 3a, 8, and E may activate

NLRP3 via potassium efflux, the strength of these studies is diminished since it is unknown if overexpression of these ORFs results in similar expression levels as during infection, as well as by overexpression of the inflammasome components themselves. My preliminary studies using pseudotyped lentivirus infection expressing ORFs 3a, 8, and E in the THP-1 monocytic cancer cell line, which endogenously express the relevant inflammasome components, did not show any role for these viroporins in inflammasome activation and pyroptosis, even following THP-1 activation with LPS or differentiation to macrophages using PMA (data not shown).

AIM2 has been shown to be activated during some RNA virus infections⁷⁷⁻⁷⁹. As a dsDNA sensor, AIM2 may also be activated by mitochondrial DNA that leaks into the cytosol following mitochondrial damage that can occur during pyroptosis⁸⁰. Thus, it seems possible that NLRP3 is activated directly by SARS-CoV-2 infection to cause pyroptosis, while AIM2 might be secondarily activated by host mitochondrial DNA released to the cytosol during pyroptosis. Live-cell imaging during pyroptosis may assist in determining which inflammasomes are activated and form specks first, and determining whether mitochondria are damaged prior to AIM2 activation. Additionally, experiments with mutant viruses could help to determine which, if any, of the putative viroporins activate the NLRP3 inflammasome by determining whether deletion of ORFs 3a, 8, or E prevents inflammasome activation and pyroptosis.

While SARS-CoV and MERS-CoV have been shown to activate inflammasomes *in vitro*, it is unknown if they activate multiple inflammasomes as we observed with NLRP3 and AIM2 during SARS-CoV-2 infection. Rare reports have previously shown colocalization of two inflammasomes within a cell¹⁰⁹, but inflammasome activation and pyroptosis during viral infection have been poorly studied, so it would be worthwhile to investigate if this occurs during other viral infections.

The discovery of a role for antibodies in enhancing monocyte infection also raises the possibility that monocyte infection could be enhanced by antibodies raised by prior infection or vaccination. Clinical deterioration coincides temporally with the detection of anti-SARS-CoV-2 antibody responses^{86–89}, and patients with severe COVID-19 have increased levels of anti-SARS-CoV-2 antibodies⁹³. Most of the patients we studied had detectable anti-spike IgG when they presented to the emergency room (data not shown). Severe COVID-19 patients also have increased IgG with afucosylated Fc regions, which allows them to bind more strongly to CD16, which may facilitate viral uptake and infection of monocytes^{110–112}. *Ex vivo* infection of healthy donor monocytes in the presence or absence of plasma from fully vaccinated individuals could be compared to infection in the presence of patient plasma to determine if antibodies from vaccinated individuals facilitate viral infection.

The question remains whether our findings of pyroptosis in monocytes can be leveraged therapeutically to improve clinical outcomes in severe COVID-19. Two FDA-approved drugs have been shown to block pyroptosis and improve survival by reducing inflammation in mouse models of sepsis^{30,31}. Both of these compounds – disulfiram and dimethyl fumarate – are cysteine reactive and prevent pyroptosis by blocking GSDMD pore formation^{30,31}. The rest of the inflammasome pathway still occurs – notably, IL-1 β processing – but since pore formation is abrogated, cytokine release does not occur. Prior attempts to prevent severe disease by antagonizing the inflammatory cytokines IL-6 and IL-1 β have had mixed results^{26–29}, but since blocking GSDMD pore formation prevents release not only of IL-1 β but also of other cytokines and cellular alarmins, upstream therapeutic intervention to inhibit the release of all inflammatory mediators may be useful in combatting severe COVID-19. Both disulfiram and dimethyl fumarate are approved for human use – as Antabuse and Tecfidera, respectively – so human

trials could be readily pursued to determine their therapeutic value in COVID-19. In fact, clinical studies of disulfiram are currently in progress in Brazil and the United States. If they prove to be effective, it will also be important to determine when they should be given. Some therapeutics, such as dexamethasone, are only effective at late stages of disease when patients show signs of severe inflammation and require supplemental oxygen²⁵. Since disulfiram and dimethyl fumarate are otherwise safe in humans and can block pyroptosis, it seems likely that they will be most effective when used early in disease before patients develop severe inflammation, which can rapidly escalate to cause vascular leak and major organ damage. Indeed, in a mouse model of sepsis, disulfiram given up to 12 hours after LPS challenge prolonged survival, though not as much as when given prior to LPS challenge³⁰.

3.4 Conclusions

The results presented in this thesis suggest that CRS seen in severe COVID-19 may be caused, at least in part, by pyroptosis of SARS-CoV-2 infected blood monocytes following activation of NLRP3 and AIM2 inflammasomes. There are still a number of unanswered questions such as what specifically causes NLRP3 and AIM2 activation and whether pyroptosis is triggered in other infected cells, but it seems likely that pyroptosis contributes to the pathology of COVID-19. This work provides a new framework for thinking about inflammation in COVID-19 and raises the possibility of preventing severe disease by targeting the upstream mediators of IL-1 family cytokine processing and release rather than trying to antagonize them following their release. Further work should elucidate the mechanisms underlying SARS-CoV-2-induced pyroptosis and determine the therapeutic potential of inhibiting pyroptosis.

Bibliography

1. Taylor, R. C., Cullen, S. P. & Martin, S. J. Apoptosis: Controlled demolition at the cellular level. *Nature Reviews Molecular Cell Biology* **9**, 231–241 (2008).
2. Liu, X. & Lieberman, J. Knocking 'em Dead: Pore-Forming Proteins in Immune Defense. *Annu. Rev. Immunol.* **38**, 455–485 (2020).
3. Cookson, B. T. & Brennan, M. A. Pro-inflammatory programmed cell death. *Trends in Microbiology* **9**, 113–114 (2001).
4. Liu, X. & Lieberman, J. A Mechanistic Understanding of Pyroptosis: The Fiery Death Triggered by Invasive Infection. in *Advances in Immunology* **135**, 81–117 (2017).
5. Dong, E., Du, H. & Gardner, L. An interactive web-based dashboard to track COVID-19 in real time. *The Lancet Infectious Diseases* **20**, 533–534 (2020).
6. Li, H. *et al.* SARS-CoV-2 and viral sepsis: observations and hypotheses. *Lancet* **395**, 1517–1520 (2020).
7. Remy, K. E. *et al.* Immunotherapies for COVID-19: lessons learned from sepsis. *The Lancet Respiratory Medicine* **8**, 946–949 (2020).
8. Moore, J. B. & June, C. H. Cytokine release syndrome in severe COVID-19. *Science* **368**, 473–474 (2020).
9. Schulte-Schrepping, J. *et al.* Severe COVID-19 Is Marked by a Dysregulated Myeloid Cell Compartment. *Cell* **182**, 1419–1440 (2020).
10. Liu, L. & Sun, B. Neutrophil pyroptosis: new perspectives on sepsis. *Cellular and Molecular Life Sciences* **76**, 2031–2042 (2019).
11. Aglietti, R. A. & Dueber, E. C. Recent Insights into the Molecular Mechanisms Underlying Pyroptosis and Gasdermin Family Functions. *Trends Immunol.* **38**, 261–271

- (2017).
12. Zhu, N. *et al.* A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N. Engl. J. Med.* **382**, 727–733 (2020).
 13. Coronaviridae Study Group of the International Committee on Taxonomy of Viruses. The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nature Microbiology* **5**, 536–544 (2020).
 14. Zhou, P. *et al.* A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature* **579**, 270–273 (2020).
 15. Hu, B., Guo, H., Zhou, P. & Shi, Z. L. Characteristics of SARS-CoV-2 and COVID-19. *Nature Reviews Microbiology* **19**, 141–154 (2020).
 16. Consiglio, C. R. *et al.* The Immunology of Multisystem Inflammatory Syndrome in Children with COVID-19. *Cell* **183**, 968–981 (2020).
 17. Del Valle, D. M. *et al.* An inflammatory cytokine signature predicts COVID-19 severity and survival. *Nat. Med.* **26**, 1636–1643 (2020).
 18. Zhou, F. *et al.* Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet* **395**, 1054–1062 (2020).
 19. Wu, C. *et al.* Risk Factors Associated With Acute Respiratory Distress Syndrome and Death in Patients With Coronavirus Disease 2019 Pneumonia in Wuhan, China. *JAMA Intern. Med.* **180**, 934–943 (2020).
 20. Lee, S. J., Channappanavar, R. & Kanneganti, T. D. Coronaviruses: Innate Immunity, Inflammasome Activation, Inflammatory Cell Death, and Cytokines. *Trends in Immunology* **41**, 1083–1099 (2020).
 21. Yan, L. *et al.* An interpretable mortality prediction model for COVID-19 patients. *Nat.*

- Mach. Intell.* **2**, 283–288 (2020).
22. Liu, X., Xia, S., Zhang, Z., Wu, H. & Lieberman, J. Channeling inflammation: Gasdermins in physiology and disease. *Nat. Rev. Drug Discov* (2021).
 23. Beigel, J. H. *et al.* Remdesivir for the Treatment of Covid-19 — Final Report. *N. Engl. J. Med.* **383**, 1813–1826 (2020).
 24. Coutinho, A. E. & Chapman, K. E. The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. *Molecular and Cellular Endocrinology* **335**, 2–13 (2011).
 25. The RECOVERY Collaborative Group. Dexamethasone in Hospitalized Patients with Covid-19. *N. Engl. J. Med.* **384**, 693-704 (2020).
 26. Novartis press release. (2020). Available at: <https://www.novartis.com/news/media-releases/novartis-provides-update-can-covid-trial-hospitalized-patients-covid-19-pneumonia-and-cytokine-release-syndrome-crs>. (Accessed: 24th February 2021)
 27. Kyriazopoulou, E. *et al.* Anakinra to prevent respiratory failure in COVID-19. Preprint at <https://www.medrxiv.org/content/10.1101/2020.10.28.20217455v1> (2020).
 28. Salama, C. *et al.* Tocilizumab in Patients Hospitalized with Covid-19 Pneumonia. *N. Engl. J. Med.* **384**, 20–30 (2021).
 29. Stone, J. H. *et al.* Efficacy of Tocilizumab in Patients Hospitalized with Covid-19. *N. Engl. J. Med.* **383**, 2333–2344 (2020).
 30. Hu, J. J. *et al.* FDA-approved disulfiram inhibits pyroptosis by blocking gasdermin D pore formation. *Nat. Immunol.* **21**, 736–745 (2020).
 31. Humphries, F. *et al.* Succination inactivates gasdermin D and blocks pyroptosis. *Science* **369**, 1633-1637 (2020).

32. Moderna TX Inc. FACT SHEET FOR RECIPIENTS AND CAREGIVERS
EMERGENCY USE AUTHORIZATION (EUA) OF THE MODERNA COVID-19
VACCINE TO PREVENT CORONAVIRUS DISEASE 2019 (COVID-19) IN
INDIVIDUALS 18 YEARS OF AGE AND OLDER. (2021). Available at
<https://www.fda.gov/media/144638/download>. (Accessed 27th April 2021)
33. Pfizer-BioNTech. FACT SHEET FOR RECIPIENTS AND CAREGIVERS
EMERGENCY USE AUTHORIZATION (EUA) OF THE PFIZER-BIONTECH COVID-
19 VACCINE TO PREVENT CORONAVIRUS DISEASE 2019 (COVID-19) IN
INDIVIDUALS 16 YEARS OF AGE AND OLDER. (2021). Available at:
<https://www.fda.gov/media/144414/download>. (Accessed: 2nd March 2021)
34. Janssen Biotech. FACT SHEET FOR RECIPIENTS AND CAREGIVERS
EMERGENCY USE AUTHORIZATION (EUA) OF THE JANSSEN COVID-19
VACCINE TO PREVENT CORONAVIRUS DISEASE 2019 (COVID-19) IN
INDIVIDUALS 18 YEARS OF AGE AND OLDER. (2021). Available at
<https://www.fda.gov/media/146305/download>. (Accessed 27th April 2021)
35. SARS | Basics Factsheet | CDC. Available at: <https://www.cdc.gov/sars/about/fs-sars.html>. (Accessed: 24th February 2021)
36. About Middle East Respiratory Syndrome (MERS) | CDC. Available at:
<https://www.cdc.gov/coronavirus/mers/about/index.html>. (Accessed: 10th December 2020)
37. Fung, S. Y., Yuen, K. S., Ye, Z. W., Chan, C. P. & Jin, D. Y. A tug-of-war between
severe acute respiratory syndrome coronavirus 2 and host antiviral defence: lessons from
other pathogenic viruses. *Emerging Microbes and Infections* **9**, 558–570 (2020).

38. Gandhi, M., Yokoe, D. S. & Havlir, D. V. Asymptomatic Transmission, the Achilles' Heel of Current Strategies to Control Covid-19. *N. Engl. J. Med.* **382**, 2158–2160 (2020).
39. Castaño-Rodriguez, C. *et al.* Role of severe acute respiratory syndrome coronavirus viroporins E, 3a, and 8a in replication and pathogenesis. *MBio* **9**, e02325-17 (2018).
40. Chen, I.-Y., Moriyama, M., Chang, M.-F. & Ichinohe, T. Severe Acute Respiratory Syndrome Coronavirus Viroporin 3a Activates the NLRP3 Inflammasome. *Front. Microbiol.* **10**, 50 (2019).
41. Nieto-Torres, J. L. *et al.* Severe acute respiratory syndrome coronavirus E protein transports calcium ions and activates the NLRP3 inflammasome. *Virology* **485**, 330–339 (2015).
42. Kern, D. M. *et al.* Cryo-EM structure of the SARS-CoV-2 3a ion channel in lipid nanodiscs. Preprint at <https://www.biorxiv.org/content/10.1101/2020.06.17.156554v3> (2020).
43. Singh Tomar, P. P. & Arkin, I. T. SARS-CoV-2 E protein is a potential ion channel that can be inhibited by Gliclazide and Memantine. *Biochem. Biophys. Res. Commun.* **530**, 10–14 (2020).
44. Siu, K. *et al.* Severe acute respiratory syndrome Coronavirus ORF3a protein activates the NLRP3 inflammasome by promoting TRAF3-dependent ubiquitination of ASC. *FASEB J.* **33**, 8865–8877 (2019).
45. Yue, Y. *et al.* SARS-Coronavirus Open Reading Frame-3a drives multimodal necrotic cell death. *Cell Death Dis.* **9**, 1–15 (2018).
46. Ahn, M. *et al.* Dampened NLRP3-mediated inflammation in bats and implications for a special viral reservoir host. *Nat. Microbiol.* **4**, 789–799 (2019).

47. Irving, A. T., Ahn, M., Goh, G., Anderson, D. E. & Wang, L. F. Lessons from the host defences of bats, a unique viral reservoir. *Nature* **589**, 363–370 (2021).
48. Kim, D. *et al.* The Architecture of SARS-CoV-2 Transcriptome. *Cell* **181**, 914–921 (2020).
49. Finkel, Y. *et al.* The coding capacity of SARS-CoV-2. *Nature* **589**, 125–130 (2021).
50. Dai, L. & Gao, G. F. Viral targets for vaccines against COVID-19. *Nature Reviews Immunology* **21**, 73–82 (2021).
51. Zoufaly, A. *et al.* Human recombinant soluble ACE2 in severe COVID-19. *Lancet Respir. Med.* **8**, 1154–1158 (2020).
52. Annunziato, F., Romagnani, C. & Romagnani, S. The 3 major types of innate and adaptive cell-mediated effector immunity. *J. Allergy Clin. Immunol.* **135**, 626–635 (2015).
53. Park, A. & Iwasaki, A. Type I and Type III Interferons – Induction, Signaling, Evasion, and Application to Combat COVID-19. *Cell Host and Microbe* **27**, 870–878 (2020).
54. Carvalho, T., Krammer, F. & Iwasaki, A. The first 12 months of COVID-19: a timeline of immunological insights. *Nat. Rev. Immunol.* **21**, 245–256 (2021).
55. Hung, I. F. N. *et al.* Triple combination of interferon beta-1b, lopinavir–ritonavir, and ribavirin in the treatment of patients admitted to hospital with COVID-19: an open-label, randomised, phase 2 trial. *Lancet* **395**, 1695–1704 (2020).
56. Monk, P. D. *et al.* Safety and efficacy of inhaled nebulised interferon beta-1a (SNG001) for treatment of SARS-CoV-2 infection: a randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet Respir. Med.* **9**, 196–206 (2021).
57. Dinarello, C. A. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood* **117**, 3720–3732 (2011).

58. Dinarello, C. A. Overview of the IL-1 family in innate inflammation and acquired immunity. *Immunological Reviews* **281**, 8–27 (2018).
59. Boucher, D. *et al.* Caspase-1 self-cleavage is an intrinsic mechanism to terminate inflammasome activity. *J. Exp. Med.* **215**, 827–840 (2018).
60. WHO. *WHO R&D Blueprint novel Coronavirus COVID-19 Therapeutic Trial Synopsis*. (2020).
61. Filbin, M. R. *et al.* Plasma proteomics reveals tissue-specific cell death and mediators of cell-cell interactions in severe COVID-19 patients. Preprint at <https://www.biorxiv.org/content/10.1101/2020.11.02.365536v2> (2020).
62. Xie, X. *et al.* An Infectious cDNA Clone of SARS-CoV-2. *Cell Host Microbe* **27**, 841-848 (2020).
63. Clark, S. A. *et al.* Molecular basis for a germline-biased neutralizing antibody response to SARS-CoV-2. Preprint at <https://www.biorxiv.org/content/10.1101/2020.11.13.381533v1> (2020).
64. Mann, E. R. *et al.* Longitudinal immune profiling reveals key myeloid signatures associated with COVID-19. *Sci. Immunol.* **5**, eabd6197 (2020).
65. Lucas, C. *et al.* Longitudinal analyses reveal immunological misfiring in severe COVID-19. *Nature* **584**, 463-469 (2020).
66. Hadjadj, J. *et al.* Impaired type I interferon activity and inflammatory responses in severe COVID-19 patients. *Science* **369**, 718–724 (2020).
67. Rodrigues, T. S. *et al.* Inflammasomes are activated in response to SARS-CoV-2 infection and are associated with COVID-19 severity in patients. *J. Exp. Med.* **218**, e20201707 (2021).

68. Liu, X. *et al.* Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. *Nature* **535**, 153–158 (2016).
69. Pascual, V., Allantaz, F., Arce, E., Punaro, M. & Banchereau, J. Role of interleukin-1 (IL-1) in the pathogenesis of systemic onset juvenile idiopathic arthritis and clinical response to IL-1 blockade. *J. Exp. Med.* **201**, 1479–1486 (2005).
70. Blanco-Melo, D. *et al.* Imbalanced Host Response to SARS-CoV-2 Drives Development of COVID-19. *Cell* **181**, 1036-1045 (2020).
71. Muñoz-Planillo, R. *et al.* K⁺ Efflux Is the Common Trigger of NLRP3 Inflammasome Activation by Bacterial Toxins and Particulate Matter. *Immunity* **38**, 1142–1153 (2013).
72. da Costa, L. S., Outlioua, A., Anginot, A., Akarid, K. & Arnoult, D. RNA viruses promote activation of the NLRP3 inflammasome through cytopathogenic effect-induced potassium efflux. *Cell Death Dis.* **10**, 346 (2019).
73. Sarkar, M. & Saha, S. Structural insight into the role of novel SARS-CoV-2 E protein: A potential target for vaccine development and other therapeutic strategies. *PLoS One* **15**, e0237300 (2020).
74. Mandala, V. S. *et al.* Structure and drug binding of the SARS-CoV-2 envelope protein transmembrane domain in lipid bilayers. *Nat. Struct. Mol. Biol.* **27**, 1202–1208 (2020).
75. Rathinam, V. A. K. *et al.* The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses. *Nat. Immunol.* **11**, 395–402 (2010).
76. Schnappauf, O., Chae, J. J., Kastner, D. L. & Aksentijevich, I. The Pyrin Inflammasome in Health and Disease. *Frontiers in immunology* **10**, 1745 (2019).
77. Spel, L. & Martinon, F. Detection of viruses by inflammasomes. *Current Opinion in Virology* **46**, 59–64 (2021).

78. Yogarajah, T., Ong, K. C., Perera, D. & Wong, K. T. AIM2 Inflammasome-Mediated Pyroptosis in Enterovirus A71-Infected Neuronal Cells Restricts Viral Replication. *Sci. Rep.* **7**, 5845 (2017).
79. Zhang, H. *et al.* AIM2 Inflammasome Is Critical for Influenza-Induced Lung Injury and Mortality. *J. Immunol.* **198**, 4383–4393 (2017).
80. Rogers, C. *et al.* Gasdermin pores permeabilize mitochondria to augment caspase-3 activation during apoptosis and inflammasome activation. *Nat. Commun.* **10**, 1689 (2019).
81. Song, X. *et al.* High expression of angiotensin-converting enzyme-2 (ACE2) on tissue macrophages that may be targeted by virus SARS-CoV-2 in COVID-19 patients. Preprint at <https://www.biorxiv.org/content/10.1101/2020.07.18.210120v1> (2020).
82. Radzikowska, U. *et al.* Distribution of ACE2, CD147, CD26, and other SARS-CoV-2 associated molecules in tissues and immune cells in health and in asthma, COPD, obesity, hypertension, and COVID-19 risk factors. *Allergy Eur. J. Allergy Clin. Immunol.* **75**, 2829–2845 (2020).
83. Wang, K. *et al.* CD147-spike protein is a novel route for SARS-CoV-2 infection to host cells. *Signal Transduct. Target. Ther.* **5**, 283 (2020).
84. Anania, J. C., Chenoweth, A. M., Wines, B. D. & MarkHogarth, P. The human FcγRII (CD32) family of leukocyte FCR in health and disease. *Frontiers in Immunology* **10**, 464 (2019).
85. Bournazos, S., Gupta, A. & Ravetch, J. V. The role of IgG Fc receptors in antibody-dependent enhancement. *Nature Reviews Immunology* **20**, 633–643 (2020).
86. Li, K. *et al.* Dynamic changes in anti-SARS-CoV-2 antibodies during SARS-CoV-2 infection and recovery from COVID-19. *Nat. Commun.* **11**, 6044 (2020).

87. Long, Q. X. *et al.* Antibody responses to SARS-CoV-2 in patients with COVID-19. *Nat. Med.* **26**, 845–848 (2020).
88. Xu, X. *et al.* Seroprevalence of immunoglobulin M and G antibodies against SARS-CoV-2 in China. *Nat. Med.* **26**, 1193–1195 (2020).
89. Tay, M. Z., Poh, C. M., Rénia, L., MacAry, P. A. & Ng, L. F. P. The trinity of COVID-19: immunity, inflammation and intervention. *Nature Reviews Immunology* **20**, 363–374 (2020).
90. Zheng, J. *et al.* Severe Acute Respiratory Syndrome Coronavirus 2-induced immune activation and death of monocyte-derived human macrophages and dendritic cells. *J. Infect. Dis.* **223**, 785–795 (2021).
91. Weber, F., Wagner, V., Rasmussen, S. B., Hartmann, R. & Paludan, S. R. Double-Stranded RNA Is Produced by Positive-Strand RNA Viruses and DNA Viruses but Not in Detectable Amounts by Negative-Strand RNA Viruses. *J. Virol.* **80**, 5059–5064 (2006).
92. Mantovani, A., Dinarello, C. A., Molgora, M. & Garlanda, C. Interleukin-1 and Related Cytokines in the Regulation of Inflammation and Immunity. *Immunity* **50**, 778–795 (2019).
93. Combes, A. J. *et al.* Global absence and targeting of protective immune states in severe COVID-19. *Nature* **591**, 124–130 (2021).
94. Aoki, T. & Narumiya, S. Prostaglandins and chronic inflammation. *Trends in Pharmacological Sciences* **33**, 304–311 (2012).
95. Aoki, T. *et al.* Prostaglandin E2-EP2-NF- κ B signaling in macrophages as a potential therapeutic target for intracranial aneurysms. *Sci. Signal.* **10**, eaah6037 (2017).
96. Robb, C. T., Goepp, M., Rossi, A. G. & Yao, C. Non-steroidal anti-inflammatory drugs,

- prostaglandins, and COVID-19. *Br. J. Pharmacol.* **177**, 4899–4920 (2020).
97. Liu, T., Zhang, L., Joo, D. & Sun, S. C. NF- κ B signaling in inflammation. *Signal Transduction and Targeted Therapy* **2**, 1–9 (2017).
 98. Lin, Y., Jamison, S. & Lin, W. Interferon- γ Activates Nuclear Factor- κ B in Oligodendrocytes through a Process Mediated by the Unfolded Protein Response. *PLoS One* **7**, e36408 (2012).
 99. Wu, C. *et al.* IFN- γ Primes Macrophage Activation by Increasing Phosphatase and Tensin Homolog via Downregulation of miR-3473b. *J. Immunol.* **193**, 3036–3044 (2014).
 100. Chang, Z. L. Important aspects of Toll-like receptors, ligands and their signaling pathways. *Inflammation Research* **59**, 791–808 (2010).
 101. Palazon-Riquelme, P. & Lopez-Castejon, G. The inflammasomes, immune guardians at defence barriers. *Immunology* **155**, 320–330 (2018).
 102. Broz, P., Pelegrín, P. & Shao, F. The gasdermins, a protein family executing cell death and inflammation. *Nature Reviews Immunology* **20**, 143–157 (2020).
 103. Robinson, K. S. *et al.* Enteroviral 3C protease activates the human NLRP1 inflammasome in airway epithelia. *Science* **370**, eaay2002 (2020).
 104. Sandstrom, A. *et al.* Functional degradation: A mechanism of NLRP1 inflammasome activation by diverse pathogen enzymes. *Science* **364**, eaau1330 (2019).
 105. Wang, Q. *et al.* CARD8 is an inflammasome sensor for HIV-1 protease activity. *Science* **371**, eabe1707 (2021).
 106. Zhu, S. *et al.* Nlrp9b inflammasome restricts rotavirus infection in intestinal epithelial cells. *Nature* **546**, 667–670 (2017).
 107. Poeck, H. *et al.* Recognition of RNA virus by RIG-I results in activation of CARD9 and

- inflammasome signaling for interleukin 1B production. *Nat. Immunol.* **11**, 63–69 (2010).
108. Xu, H. *et al.* SARS-CoV-2 viroporin triggers the NLRP3 inflammatory pathway. Preprint at <https://www.biorxiv.org/content/10.1101/2020.10.27.357731v1> (2020).
109. Swanson, K. V. *et al.* A noncanonical function of cGAMP in inflammasome priming and activation. *J. Exp. Med.* **214**, 3611–3262 (2017).
110. Iwasaki, A. & Yang, Y. The potential danger of suboptimal antibody responses in COVID-19. *Nature Reviews Immunology* **20**, 339–341 (2020).
111. Chakraborty, S. *et al.* Proinflammatory IgG Fc structures in patients with severe COVID-19. *Nat. Immunol.* **22**, 67–73 (2021).
112. Larsen, M. D. *et al.* Afucosylated IgG characterizes enveloped viral responses and correlates with COVID-19 severity. *Science* **371**, eabc8378 (2021).