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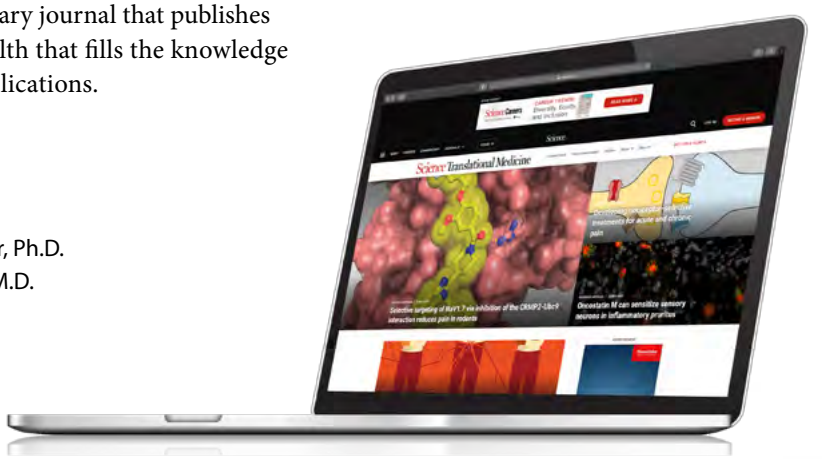
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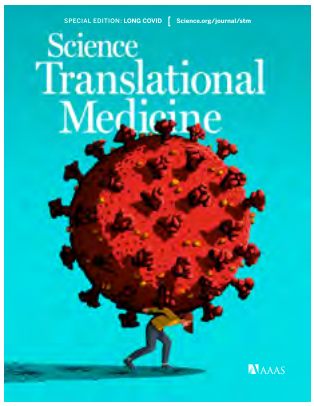
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## SPECIAL EDITION: LONG COVID




The cover image symbolizes the burden that individuals with Long Covid bear as a consequence of infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2, red virion). This Special Issue highlights our current knowledge of Long Covid and other infection-associated chronic conditions (IACCs), emphasizing the work required to improve outcomes for the millions of people worldwide affected by IACCs. An Editorial by Marrazzo *et al.* describes the goals and impact of the NIH RECOVER Initiative for improving diagnosis and treatment of Long Covid. Four Viewpoints discuss the long-term effects of COVID-19 in children (Saydah *et al.*), how Long Covid informs our understanding of other IACCs (Peluso *et al.*), sex-specific differences in Long Covid (Silva and Iwasaki), and how another IACC, posttreatment Lyme disease, is shedding light on IACC diagnosis and treatment (Marques). One Review article discusses animal models of Long Covid (Schäfer *et al.*), and another Review provides a comprehensive analysis of potential drivers of and candidate therapeutics for Long Covid (Antar and Cox). A Research Article reporting how sex-specific differences during acute COVID-19 correlate with Long Covid development and resolution (Hamlin *et al.*) completes this Special Issue.

Credit: Stephan Schmitz (Folio Art)

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LONG COVID

# Initiating Long Covid RECOVERy

**The NIH's RECOVER Initiative aims to ease the suffering of those living with Long Covid.**



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The coronavirus disease 2019 (COVID-19) pandemic paralyzed the United States, rendering thousands critically ill and ultimately killing more than 1 million Americans. Many survivors, particularly those with adult respiratory distress syndrome, required prolonged rehabilitation. Many more people, including those who did not require hospitalization for their acute illness, presented with a host of other persistent, disabling symptoms. The latter condition was termed "Long Covid" and turned out to be the most prevalent postacute sequelae of the COVID-19 pandemic. The symptom complex that characterizes Long Covid resembles that seen in other infection-associated chronic conditions, notably overlapping with those of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS). Overlapping symptoms include fatigue, unrefreshing sleep, neurocognitive dysfunction characterized by impaired executive function, exercise intolerance, fluctuating heart rate and sense of dizziness particularly in the upright position, and postexertional malaise, a signature symptom of ME/CFS. The drivers of these conditions remain unknown, and no treatments have proven effective. Data suggest that many individuals with Long Covid may return to health months or years after onset, but debilitating symptoms and unknown long-term outcomes remain in too many people. Of greatest concern is that, for some individuals, Long Covid may last a lifetime.

In December of 2020, the leadership of the National Institutes of Health (NIH) held a workshop to discuss launching research to improve understanding of what was then referred to as the postacute sequelae of COVID-19 (PASC), which came to be known as Long Covid, and the underlying causes. The US Congress realized the threat to public health that Long Covid presented and appropriated $1.15 billion to the NIH for more than 4 years to support research to understand, prevent, diagnose, and treat the condition through an initiative that was named Researching COVID to Enhance Recovery (RECOVER).

At its onset, recognizing the tremendous scope of the problem, RECOVER-funded investigators across the country embarked on studies to discover important pathophysiological processes at play in individuals with Long Covid (recovercovid.org). From the beginning, RECOVER was built on a national scale, with research teams using similar protocols so that results could be shared and compared to get robust answers quickly. Several reports from NIH-funded investigators, as well as scientists around the world, identified a series of abnormalities, including viral persistence, antigen persistence, reactivation of latent viruses, autoimmunity, immune dysfunction, and clotting abnormalities. Whether these are cause or effect, whether they generalize across all individuals with Long Covid or only a subset, and whether they can be replicated in larger cohorts are features yet to be determined. However, RECOVER has provided a firm foundation for focused follow-up studies.

RECOVER has five major components: longitudinal cohort studies, pathobiology studies, electronic health records research, autopsy and tissue pathology studies, and clinical trials (recovercovid.org/research-components). The adult cohort consists of approximately 15,200 individuals, including roughly 2200 pregnant women who were infected with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The pediatric cohort is composed of more than 24,000 babies, children, and young adults. These individuals provide clinical data, and all participants undergo the same initial testing (tier 1). Tier 2 and tier 3 testing was devised to delve even deeper into specific symptom clusters with imaging, neurocognitive, autonomic, and other testing to complete the clinical picture. Thus far, the adult cohort has donated more than 822,000 biospecimens to the RECOVER biorepository, whereas the pediatric cohort has donated more than 85,000 biospecimens (recovercovid.org/data).

The clinical data and matched samples are invaluable for discovering the biological abnormalities underlying Long Covid and as resources to validate findings from smaller studies around the world. RECOVER also launched a program to obtain autopsy tissue from people who died months or years after their SARS-CoV-2 infection for in-depth analyses searching for clues to the pathophysiology that could not be uncovered in living individuals. As quickly as possible, RECOVER data and samples are made available to researchers, even those not currently affiliated with RECOVER. Additionally, RECOVER electronic health records research leveraged records of 60 million individuals across the country, yielding findings that provided some of the earliest insights into Long Covid.

A debt of gratitude is owed to the volunteers who enrolled in the study and their caregivers, as well as the researchers and health professionals who informed the study's design. Many if not most participants are people with lived experience of Long Covid, ME/CFS, and other infection-associated chronic conditions.

People affected by Long Covid have been at the center of RECOVER from the very beginning, playing critical roles in designing studies, responding to surveys, serving on governance and publication groups, and

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guiding the initiative. Patient representatives were integral to the design of the clinical trials and worked closely with RECOVER researchers and health care providers to ensure that the trials addressed safety concerns raised by people affected by Long Covid.

Most symptoms experienced by people with Long Covid are not unique and fall into specific symptom clusters. Investigators devised and tested an algorithm based on symptoms that distinguishes those with Long Covid from the general population (*1*). Similar characterization of Long Covid came from RECOVER's pediatric cohort study. Electronic health records research from RECOVER reported the effectiveness of vaccination in reducing the risk of Long Covid and that Long Covid is associated with risk factors such as being middle-aged (between 40 and 69 years of age), being female, and having certain chronic health conditions (such as obesity or lung disease) before having COVID-19 (*2*, *3*). It also stands to reason that communities disproportionately affected by COVID-19 may be more likely to get Long Covid, rendering racial and ethnic minority communities with higher odds of developing the condition. With more than 45 peer-reviewed publications and more in progress, the RECOVER Initiative is poised to continue yielding important findings, many of which may suggest potential treatment targets (recovercovid.org/publications).

To date, RECOVER has designed a series of five symptom-based clinical trial platforms to launch multiple interventions (trials.recovercovid.org). Four of these platforms were designed for specific symptom clusters that were identified in the RECOVER cohort and confirmed by people with lived experience as symptoms that are most disabling and burdensome. These include protocols to test interventions in individuals with autonomic symptoms, disordered sleep, exercise intolerance or postexertional malaise, and neurocognitive dysfunction. The fifth platform, RECOVER VITAL, was built to test interventions that might treat viral persistence, hypothesized as a root cause of Long Covid. A 2022 study reported persistence of SARS-CoV-2 in autopsy cases up to 230 days from initial infection, raising the possibility that Long Covid might be due to inadequate clearance of virus (*4*). The first VITAL study is prolonged treatment (up to 28 days) with oral nirmatrelvir/ritonavir to clear the remaining virus. It remains to be determined whether there is a means to identify those who do or do not have persistent virus, so the current study includes participants based on symptoms. The protocol, however, can adapt to test multiple treatments aimed at the root causes of Long Covid, whether they be persistent or reactivated virus, immune dysregulation, or autoimmune disease. Importantly, the collaboration between VITAL, FNIH, FDA, and industry partners has yielded a better definition of regulatory pathways for approval of new treatments for Long Covid. RECOVER's AUTONOMIC protocol is testing intravenous gamma globulin, effective in chronic autoimmune neuropathies, and ivabradine, a drug that lowers heart rate. RECOVER NEURO is testing a brain training program, an online goal management program, and noninvasive brain stimulation toward improving cognitive dysfunction symptoms. RECOVER ENERGIZE will examine the effectiveness of cardiopulmonary rehabilitation for exercise intolerance and, for individuals who are experiencing postexertional malaise, a structured pacing protocol for symptom management. RECOVER SLEEP will test interventions for Long Covid–related changes in sleep patterns. One trial is testing the US Food and Drug Administration–approved neurostimulants modafinil and solriamfetol for hypersomnia, and another is testing interventions such as melatonin and light therapy to improve sleep disturbances. Altogether, these five platforms are testing 13 interventions in eight clinical trials simultaneously and are built to continue testing until solutions are found for those still suffering. Looking to the future, we fully recognize the challenges in uncovering the cause of Long Covid, including the broad spectrum of recovery phenotypes and the myriad social determinants of health that influence Long Covid risk and outcomes. Although it is gratifying that data show continued recovery of many individuals even after suffering for 2 to 3 years after their acute infection, critical work must continue to ensure that thousands do not progress to a lifelong illness resembling ME/CFS.Infection-associated chronic conditions remain complex, challenging, and perhaps one of the least understood areas of medicine. However, there remains hope that the efforts of research participants and scientists, throughout the United States and around the world, to understand and treat Long Covid may yield discoveries that generalize to other infection-associated chronic conditions like ME/CFS.

Achieving public health success will require rigorous study of the potential culprits by investigators across the country. To this end, NIH and the biomedical community have begun mounting and coordinating major efforts across multiple scientific disciplines. The field will need to subject compelling hypotheses to rigorous validation studies and then rigorous clinical trials. Like medical mysteries of the past, this one too will be solved by science. How fast that occurs depends on continued attention and robust and rigorous scientific pursuit.

**— Jeanne Marrazzo, Gary H. Gibbons, Walter Koroshetz**



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Submitted 24 July 2024
Accepted 21 October 2024
Published 13 November 2024
10.1126/scitranslmed.adr9971

LONG COVID

# Consequences beyond acute SARS-CoV-2 infection in children

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**Sharon H. Saydah[1], Angela P. Campbell[1], Adrienne G. Randolph[2,3]***

**Although most children are spared from developing complications from SARS-CoV-2 infection, some may suffer consequences including Long Covid and multisystem inflammatory syndrome in children (MIS-C). Although the occurrence of these conditions has decreased over time, they can still occur, and recognition of symptoms and prompt diagnosis is imperative for early intervention.**

## INTRODUCTION

Millions of children have been infected with severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2), and most had infections with no or mild symptoms (*1*). Of the many thousands of children who developed more severe coronavirus disease 2019 (COVID-19), many were hospitalized, and a small percentage died. Most SARS-CoV-2–infected children recover fully, but some develop post-acute sequelae such as Long Covid or multisystem inflammatory syndrome in children (MIS-C). Here, we compare these two types of complications affecting children, including their unique presentations and usual clinical course (Fig. 1A).

## LONG COVID

In the United States, Long Covid refers to a range of physical and mental health symptoms that are present for at least 3 months after SARS-CoV-2 infection (Fig. 1A) (*2*). Symptoms and conditions may persist from the initial infection, reoccur, or emerge months later. Long Covid is defined similarly in adults and children (*2*–*5*). Many terms refer to Long Covid, including post-COVID conditions (PCCs) and post-acute sequelae of SARS-CoV-2 (PASC). Long Covid includes pathophysiological effects resulting in newly identified health conditions affecting one or many organ systems (*3*, *5*, *6*). Long Covid also encompasses symptoms with unclear pathology and features, similar to other postinfectious syndromes, including myalgic encephalomyelitis/chronic fatigue syndrome, dysautonomia, and postural orthostatic tachycardia syndrome (*3*). Across countries and studies, the definitions of Long Covid may differ regarding symptom spectrum, duration, and persistence (*7*), but there is general agreement that Long Covid negatively affects health and quality of life.

Estimated from population-based studies, 1.3% of US children have been reported to have Long Covid (*5*), with a broad spectrum of symptoms. Reports of the occurrence of Long Covid vary across studies because of differing definitions (broad or specific), study designs (cross-sectional or prospective cohort), and populations (community-based, outpatient, or hospitalized) (*4*, *8*). Long Covid occurs more frequently in all ages after more severe acute COVID-19, with more than one in four children developing it after critical illness (*9*).

Although older children are reportedly at higher risk of Long Covid than young children, this could be influenced by younger children's inability to describe their symptoms or by lack of symptom recognition by caregivers. Definitions of abnormal are determined in the context of developmental milestones, given that sleeping, eating, and behavioral patterns differ by age. Given these limitations, the symptoms most commonly reported by children diagnosed with Long Covid include increased respiratory effort, fatigue, loss of smell (anosmia) or taste (dysgeusia), chest pain, headache, lightheadedness, and tiredness or sleepiness (*8*, *10*, *11*). Often these symptoms affect childrens' daily activities, including the ability to participate in school or sports activities. Some of these symptoms or conditions may occur among children regardless of preceding SARS-CoV-2 infection, making attribution to infection difficult (*3*). In addition to new or ongoing symptoms, SARS-CoV-2 infection can increase the risk of newly diagnosed conditions after acute illness such as myocarditis or diabetes (*12*). Although many of these conditions are generally uncommon among children, there is an increased risk among those who had COVID-19 (e.g., 200% increased risk of myocarditis and 20% increased risk of type 1 diabetes) compared with those who did not have COVID-19 (*3*).

Multiple mechanisms underlying the pathophysiology of Long Covid have been proposed, including ongoing immune dysregulation, autoimmunity, microvascular damage, inflammation, and viral persistence (*6*). Whether the pathophysiology of Long Covid differs among children and adults is unclear, and large national studies such as Researching COVID to Enhance Recovery (RECOVER, NCT05172011) are ongoing to understand these mechanisms (*13*). Long Covid respiratory symptoms are more common in individuals with chronic respiratory conditions such as asthma, and fatigue and failure to resume baseline activities after developing severe COVID-19 are more common in obese children (*9*). Children with a history of immunological conditions or allergies may also have an increased risk of Long Covid (*6*, *7*). One reason Long Covid may be less frequent in children and adolescents is that, on average, they have fewer risk factors than adults, including underlying medical conditions and obesity.

Because more severe COVID-19 is associated with persistent symptoms, it is hypothesized that early treatment of COVID-19 with anti–SARS-CoV-2 or anti-inflammatory interventions could potentially decrease the risk of developing Long Covid (Fig. 1B). Vaccination is likely the key to preventing Long Covid in children (*13*), in part by decreasing the risk of developing a severe acute infection (*14*, *15*). Clinicians and caregivers should be aware of the potential for occurrence

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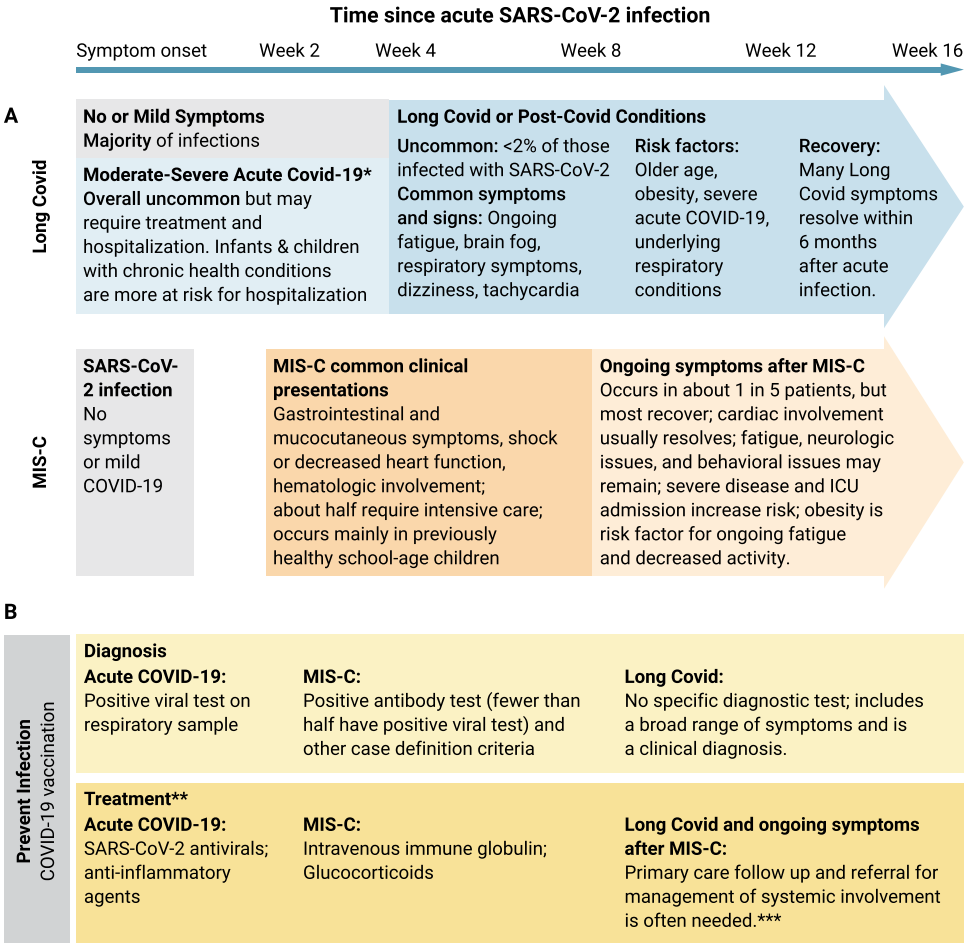



**Fig. 1. A comparison of the timing, clinical characteristics, and outcomes of Long Covid and MIS-C.** (**A**) Shown are timelines for the development of Long Covid and MIS-C after initial SARS-CoV-2 infection. *The size of the "Moderate-Severe Acute COVID-19" box does not correspond to the proportion of children and adolescents who have moderate-severe acute COVID-19. The majority of children with COVID-19 have mild or no symptoms. (**B**) Shown are approaches to diagnose, prevent, and treat acute COVID-19, MIS-C, and SARS-CoV-2. **Not all patients with acute COVID-19 are recommended to receive targeted treatment. Treatment for severe acute COVID-19 includes SARS-CoV-2 antiviral therapy, steroids, and anticytokine antibody therapies. Treatment for MIS-C includes IVIG, glucocorticoids, and anticytokine antibody therapies. In both, anticytokine antibody therapies are usually reserved for patients not responding to other interventions. ***Specialist care might include cardiology (especially for MIS-C), neurology, rheumatology, infectious disease, pulmonology, and rehabilitation. ICU, intensive care unit.

of Long Covid in the months after SARS-CoV-2 infection and monitor children for relevant signs and symptoms. Follow-up may require multidisciplinary expertise given the range of potential complications (*16*).

## MIS-C

MIS-C is a rare but serious hyperinflammatory complication that emerged worldwide during the COVID-19 pandemic, affects children and adolescents, and generally occurs 2 to 6 weeks after acute SARS-CoV-2 infection (*17*). In contrast with the preceding infection, which is usually asymptomatic or mild, MIS-C is a severe health complication requiring hospitalization. Case definitions for MIS-C standardly require dysfunction in two or more organ systems, elevated inflammatory markers, and laboratory evidence of a prior SARS-CoV-2 infection or an epidemiological link to a COVID-19 case (*17*). Sociodemographic factors initially appeared to be risk factors for MIS-C, but associations were not confirmed in later analyses; however, genetic risk factors have been identified in some patients, including carriage of rare hyperinflammatory gene variants (*18*). In one case-control analysis, compared with community controls who did not develop MIS-C but who also had tested positive for SARS-CoV-2, increased likelihood of MIS-C was associated with caregiver-reported factors that increased exposure to SARS-CoV-2, such as household crowding, large event attendance, limited masking at school, public transit use, and positive household cases, with risk increasing with the number of factors reported (*19*).

Because of the post-acute timing of MIS-C after SARS-CoV-2 infection and usual presentation with acute critical illness (Fig. 1A), MIS-C is generally considered unique from Long Covid. The clinical presentation of children with MIS-C is often initially severe, with approximately half requiring admission to the intensive care unit often because of cardiovascular involvement with shock or decreased cardiac function visualized on echocardiography. Although there is some evidence that treatment with glucocorticoids

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alone may be sufficient in some patients (*20*, *21*), first-line treatment for most children hospitalized with MIS-C has included intravenous immunoglobulin (IVIG) and glucocorticoids (Fig. 1B) (*17*, *22*). Initial treatment with both was associated internationally across three cohorts with more rapid resolution of cardiovascular symptoms in severe cases (*23*). Because children with MIS-C often develop cardiac complications such as decreased function, arrhythmias, or coronary artery aneurysms, cardiac follow-up is important to ensure resolution (*22*, *24*) given that acute myocarditis was still present in some patients on cardiovascular magnetic resonance imaging ~1 month after symptom onset (*25*).

Fortunately, most patients with MIS-C are usually discharged from the hospital within a week with rapid resolution of inflammation, shock, and cardiac dysfunction (*22*). Although most report returning to baseline within 2 to 4 months after MIS-C diagnosis, approximately one-fifth of children with MIS-C may have ongoing fatigue and other symptoms that impair their ability to return to baseline activities, similar to Long Covid (*9*). Persistent symptoms and activity impairments 2 to 4 months after MIS-C hospitalization are associated with having more severe acute illness requiring life-supportive interventions and with preexisting comorbidities, including asthma and obesity (*9*).

Acute severe neurological complications are uncommon in patients hospitalized with MIS-C, but neuropsychological sequelae may occur. In a study that compared in-depth neuropsychological assessments 6 to 12 months after hospital discharge in patients with MIS-C with those of sibling or community controls, patients with MIS-C had worse working memory, higher scores for depression and somatization, and lower quality of life scores (*26*). Another 6-month post–MIS-C follow-up study reported emotional difficulties in approximately 20% of children (*27*). Therefore, it is important to monitor children after hospital discharge for MIS-C for neurological and psychological symptoms.

The mechanisms leading to immune dysregulation manifesting as the acute life-threatening hyperinflammatory condition called MIS-C are unclear for most children (*18*). Recently, molecular mimicry from autoantibodies was reported in some patients (*28*). Fortunately, MIS-C is now even rarer than it was early in the pandemic (*1*, *29*). This is likely because of development of immunity from acute SARS-CoV-2 infection and from COVID-19 vaccination (*30*–*32*) but also may be related to changing circulating SARS-CoV-2 variants (*1*).

## CONCLUSIONS

Although children are less likely than adults to develop sequelae after SARS-CoV-2 infection, complications sometimes occur, including Long Covid and MIS-C. Developmental-associated immune differences may explain why SARS-CoV-2 affects children differently than adults (*1*, *18*). Although post–SARS-CoV-2 complications are uncommon in children, identifying them when they occur and intervening early may ensure optimal longer-term health outcomes. Vaccination is likely the best method for prevention of consequences beyond SARS-CoV-2 infection in children; no other interventions have been strongly associated with reducing the risk of developing Long Covid or MIS-C (*14*, *30*–*32*). Waning immunity after vaccination and ongoing emergence of new SARS-CoV-2 variants require receipt of updated COVID-19 vaccines at regular intervals to provide protection against SARS-CoV-2–related sequelae (*15*). Because sequelae of SARS-CoV-2 infection can affect the social, intellectual, and emotional development of children, identifying other effective interventions is essential.

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**Acknowledgments**
**Funding:** This work is supported by a Centers for Disease Control and Prevention (CDC) contract to Boston Children's Hospital to direct studies related to complications of SARS-CoV-2 in children (to A.G.R.). S.H.S. and A.P.C. are CDC employees. The findings and conclusions in this report are those of the author(s) and do not necessarily represent the official position of the CDC. **Author contributions:** S.H.S., A.P.C., and A.G.R. wrote the article and prepared the figure. **Competing interests:** The authors declare that they have no competing interests.

10.1126/scitranslmed.ado2099

LONG COVID

# Infection-associated chronic conditions: Why Long Covid is our best chance to untangle Osler's web

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**The recognition of Long Covid has renewed efforts to understand other infection-associated chronic conditions (IACCs). Here, we describe how studies of Long Covid and other IACCs might inform one another. We argue for the importance of a coordinated research agenda addressing these debilitating illnesses.**

## INTRODUCTION

For nearly a century, individuals with medically unexplained chronic conditions, particularly those thought to be attributable to presumably transient infectious pathogens, have faced bewilderment, skepticism, or outright dismissal from the medical establishment. Debilitating symptoms lasting for years have been reported after acute infections with viruses [enterovirus, Epstein-Barr virus (EBV), influenza virus, Ebola virus, dengue virus, chikungunya virus, West Nile virus, and severe acute respiratory syndrome coronavirus 1 (SARS-CoV)], bacteria (*Borrelia* and *Anaplasma*), and protozoa (*Giardia*) (*1*). Myalgic encephalomyelitis (ME), sometimes referred to as chronic fatigue syndrome (CFS), is perhaps the best example of a disabling syndrome that many experts believe follows an acute, often undiagnosed viral infection. Several names have been applied to these syndromes, including post-acute infection syndromes (PAIS), infection-associated chronic illnesses, and infection-associated chronic conditions (IACCs). Here, we will use IACCs.

Despite consistent reports regarding these conditions dating back nearly 100 years (*2*–*4*), the biomedical establishment has made limited progress in defining the epidemiology, natural history, and pathogenesis of most IACCs. No diagnostic tests are available, no widely accepted treatments exist, and industry engagement on finding a cure has been limited. In her 1996 book *Osler's Web*, investigative journalist Hillary Johnson catalogued the challenges facing ME/CFS research (*5*), which can be applied to many IACCs. Barriers to progress included the inability to fit ME/CFS into existing disease paradigms, variability and inconsistency in case ascertainment, skepticism on the part of many clinicians and scientists, and intense stigma that kept many of those affected from seeking medical care. William Osler, the "father of modern medicine," emphasized the importance of listening to patients to discern important features of their condition. However, contemporary medical practice relies heavily on diagnostic tests, which are currently inadequate to confirm the presence of an IACC. This results in people being neglected or misdiagnosed and prevents them from receiving appropriate care and support.

The year 2020 has the potential to be a turning point in this story. Shortly after the COVID-19 pandemic began, reports of individuals with prolonged COVID-attributed symptoms emerged, a condition now often referred to as Long Covid. The synchronicity of the inciting infection, universality of the exposure, and visibility, aided by social, popular, and scientific media (*6*), resulted in the ideal environment for a coordinated effort to understand this new IACC. Substantial investment in scientific effort is starting to pay off, with real progress in defining the epidemiology, natural history, and biology of Long Covid now emerging. After a Congressional appropriation, the US National Institutes of Health rapidly launched the Researching COVID to Enhance Recovery (RECOVER) initiative, which is the first large-scale program aimed at tackling an IACC. The progress to date has been hard-won, however, in part because there is no widely accepted clinical definition, biomarker, or diagnostic test for Long Covid. However, clinical trials, slow to start, are now being pursued in earnest. Although there is no guarantee that this momentum will be sustained without dedicated scientific and financial commitments (*7*), there is reason to believe that efforts to understand Long Covid have the potential to draw attention to, reframe, and revitalize the efforts to study other IACCs.

High-quality academic reviews of Long Covid are multiplying rapidly (*8*–*12*). Our goal in this Viewpoint is not to provide a comprehensive overview of the field but rather to place efforts to study Long Covid in the context of other IACCs. In doing so, we hope to outline several areas that we believe will require consideration as the field attempts to make progress in navigating what has been described as a "labyrinth" (*5*).

## CHALLENGES IN THE STUDY OF INFECTION-ASSOCIATED CHRONIC CONDITIONS

For decades, chronic conditions after clear infectious triggers have been recognized (*1*). Many survivors of the 2014–2016 Ebola outbreak in West Africa reported symptoms persisting for years (*13*). Similar outcomes were documented among survivors of the first SARS-CoV pandemic (*14*), foretelling the far more massive burden of disease that has emerged in the wake of COVID-19. Although viruses are the pathogens most implicated in IACCs, chronic and posttreatment Lyme disease are examples of chronic conditions triggered by a bacterial infection (*15*, *16*). Similarly, a 2004 Norwegian outbreak of the waterborne parasite *Giardia duodenalis* resulted in a high proportion of post-acute fatigue and abdominal symptoms (*17*). Many individuals with ME/CFS attribute their symptom onset to mononucleosis or an unknown viral-like illness (*18*). Some outbreaks of ME/CFS have occurred concurrently with known virus epidemics, including poliovirus outbreaks (*2*). During the 1980s, multiple outbreaks of ME/CFS resulted in a major increase in public awareness of the condition (*2*, *3*, *5*, *19*), but without identification of the pathogen responsible, skeptical medical professionals and negative popular

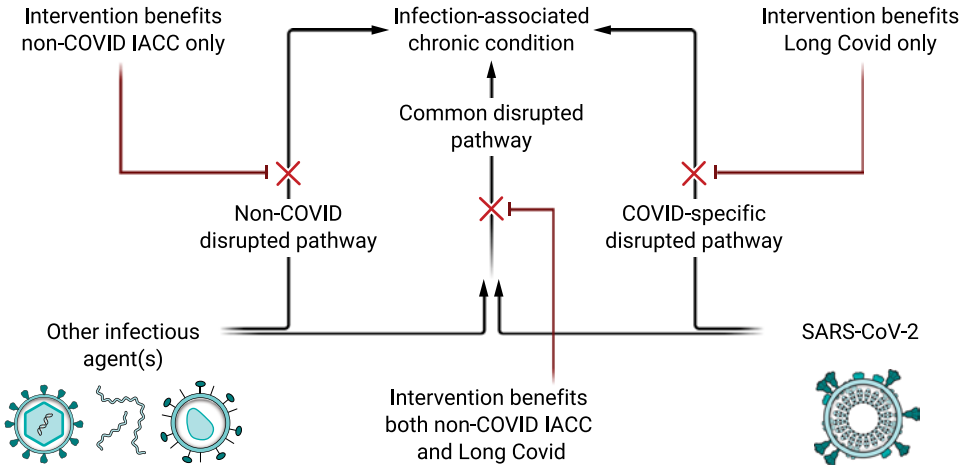
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media portrayals of those affected resulted in dismissal of the illness as unimportant and its infectious nature doubted. The Centers for Disease Control (CDC) investigation of an outbreak affecting several hundred people in Incline Village, Nevada is now seen as a missed opportunity to identify the inciting pathogen for ME/CFS (*5*). Even now, with the virus that incites Long Covid actually known, patients are often unable to access medical care because of the skepticism of some health care professionals confronted with an unfamiliar syndrome, as well as insufficient specialty clinics to meet the demand (*20*, *21*). Although the inciting pathogens must differ, the clinical presentations of ME/CFS, certain other IACCs, and some phenotypes of Long Covid are remarkably similar, suggesting that there could be at least some overlapping biological pathways (Fig. 1).

Inherent in the study of all IACCs are several key challenges. In the absence of a large-scale outbreak or a severe disease in the acute phase, many patients and clinicians may assume that a person with a mild viral-like illness will recover. As a result, tests to identify the pathogen are unlikely to be sought or performed. By the time it is recognized that an IACC has developed, it is likely to be long after the acute phase of illness, when routine tests might have identified a pathogen. Thus, most individuals are unable to attribute their illness onset to a specific infectious trigger. In contrast, Long Covid emerged in the context of a pandemic virus. Many of the SARS-CoV-2 infections to which Long Covid is attributed were clearly documented, and the reduction in other circulating pathogens (e.g., influenza, rhinovirus, and other common cold coronaviruses) in the first years of the pandemic makes the attribution less murky. The single greatest distinction between Long Covid and most other IACCs is the scale, specificity, and immunologic novelty of the responsible organism, making case ascertainment relatively straightforward in the early COVID-19 pandemic era of frequent testing and high-quality public health surveillance.

Asymptomatic infection is also a major challenge. Given the high prevalence of asymptomatic SARS-CoV-2 infection and recent reductions in testing, the ability to trace any "long" syndrome to a prior SARS-CoV-2 infection will become difficult, hindering both care and research. Serologic tests that would only be positive in those previously infected with SARS-CoV-2 could be helpful in this regard; for example, infection but not vaccination will induce a positive nucleocapsid antibody response, although variability in the magnitude and durability of such responses limit the utility of now available tests (*22*–*24*); interferon-γ release assays could play a role, but their availability is now limited (*25*). In addition, with the re-emergence of other culprit viruses, some people who develop a condition consistent with Long Covid may actually have an IACC caused by a different pathogen, even if they have evidence of prior SARS-CoV-2 infection. A similar challenge exists with Lyme disease, especially in areas where tickborne illness is endemic. Although the identities of the pathogens that incited the 1980s ME/CFS outbreaks were never determined, abundant evidence from earlier outbreaks as well as sporadic cases strongly implicates members of the enterovirus family (*3*). This large and diverse family of viruses is cited by the CDC as the cause of as many as 15 million infections each year in the US, with many of them asymptomatic (*26*). Given that SARS-CoV-2 is now likely to circulate indefinitely, and surges associated with new variants will be common but perhaps not confirmed by testing given limited investment in case ascertainment, the emergence of "sporadic" Long Covid will make it challenging to distinguish it from other IACCs.

Although people with Long Covid still face skepticism, the fact that this syndrome emerged in the context of a recognized pandemic with high morbidity and mortality provided the possibility of avoiding many of the obstacles faced by people with other IACCs. Imagine that instead of a novel coronavirus spreading globally in 2020, one of the four common cold coronaviruses mutated and resulted in an illness with chronic post-acute symptoms. Given the unmemorable nature of the acute phase illness, those who months later experienced chronic symptoms might be unable to recall the inciting infection, potentially leading medical professionals and others to incorrectly diagnose a psychological illness or even accuse the person of malingering to avoid work and other responsibilities, a fate that has befallen many with IACCs, especially ME/CFS. Instead, Long Covid emerged from an extraordinary worldwide pandemic, when everyone was paying attention to the inciting virus and patients were able to join together to draw attention to the issue (*27*). It therefore represents the best opportunity to




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**Fig. 1. Shared and pathogen-specific mechanistic pathways may both drive IACCs.** An intervention that disrupts a shared pathway would be expected to benefit both non-COVID and COVID-specific conditions, but other pathways might be unique to a specific IACC.

figure out the natural history, pathogenesis, and management of an IACC.

## WHAT CAN LONG COVID RESEARCHERS LEARN FROM 40 YEARS OF IACC STUDIES?

Despite serious underfunding, important information has emerged from the many years of studying ME/CFS and other IACCs that is relevant to efforts to understand Long Covid. The first is the importance of defining the syndrome under study. The diagnostic criteria for ME/CFS have evolved over time, and, in the absence of definitive molecular or biochemical assays, a set of particular symptoms must be used to define the illness (*28*). Criteria that are too general will result in inclusion of individuals whose underlying abnormalities are not shared, making it difficult to identify biomarkers and draw conclusions. Although still controversial, the criteria that have achieved the most acceptance include a requirement for postexertional malaise (PEM)—such as the Institute of Medicine diagnostic criteria (*28*), the Canadian Consensus Criteria (*29*), and the International Consensus Criteria (*30*).

A similar exercise now needs to be done for Long Covid [several are underway, including one recently completed by the National Academies of Sciences, Engineering, and Medicine (*31*)]. Many individuals with severe cases of COVID-19 have verifiable organ damage that results in well-recognized and easy-to-define complications, such as lung damage causing dyspnea or endothelial inflammation leading to cardiovascular events (*32*). These outcomes are an important part of the research agenda but in many cases are distinct and largely unrelated to the syndrome that is Long Covid. Lumping individuals who, for example, have sequelae from ventilator-induced lung injury with those who had mild illness followed by unexplained symptoms could confound understanding of the biological basis of the continued symptoms.

Just as there are distinct endotypes of ME/CFS and other IACCs, individuals with Long Covid may need to be separated into distinct symptom clusters (*33*), despite all cases being driven by SARS-CoV-2 infection. The reasons for these diverse symptom manifestations are unknown. Although this heterogeneity could be driven by different variants, preexisting immunity, or treatment approaches, it is important to note that distinct endotypes of Long Covid emerged in 2020, long before any of these confounders. Likewise, in ME/CFS, it is likely that different members of the enterovirus family were responsible for different outbreaks, given the rapid evolution of enteroviruses and the temporal and geographical separation of the outbreaks. Nevertheless, there are many other sources of variation in presentation that need to be studied, including which latent herpesviruses a patient may be carrying, their environmental exposures, their microbiomes, and especially their genetics and epigenetics.

A major source of variation between cases, which is common to most IACCs, is biological sex. Biological females experience many of these illnesses at least two or three times as frequently as males. Elucidating the role of sex will go a long way to addressing stigma and might lead to sex-specific strategies for prevention and treatment of IACCs. Also, it seems highly likely that the sex effect is providing a clue regarding some fundamental pathway common to most or all IACCs. Given the role of sex as a contributing factor to autoimmunity and inflammation-associated diseases (e.g., systemic lupus erythematosus and multiple sclerosis), it seems likely that immune dysfunction will prove to be a key part of the answer. The sex-based dichotomy in Long Covid and other IACCs should be a major area of research focus in the coming years.

## SHOULD LONG COVID AND OTHER IACCS BE STUDIED TOGETHER?

Many symptoms overlap between Long Covid after 2020 and ME/CFS cases before 2020 (*34*). These similarities have led some to propose that the two conditions be considered one and the same, and some people with Long Covid who fulfill at least some ME/CFS diagnostic criteria are being told they have ME/CFS. Early efforts have suggested that a high proportion of those with Long Covid meet ME/CFS criteria (*35*), but many of these studies are limited by nonrepresentative cohorts, lack of pre-COVID symptom data, and incomplete case definitions applied post hoc. Together, these factors make most prevalence estimates to date uninterpretable. Fully addressing this question will require well-characterized participants, clear attribution of symptoms to a SARS-CoV-2 infection, and prospective assessment beginning in the acute phase. Such efforts are now underway through large studies like the RECOVER initiative.

Although there may be overlap between Long Covid and other IACCs, assuming that all Long Covid is ME/CFS (or that all Long Covid is driven by postural orthostatic tachycardia or mast cell activation, for example) is also scientifically problematic, because it dismisses what is perhaps the most important lead in our efforts to understand Long Covid: That we can often identify the culprit virus. The distinction will be critical in research studies that aim to include both groups, allowing for the assessment of whether any mechanistic pathways are shared or whether they might be specific to either SARS-CoV-2 or ME/CFS or other conditions (Fig. 1). The risks of conflating Long Covid and ME/CFS go both ways; although there is hope that Long Covid research will provide some of the answers needed to fully understand ME/CFS, this should not come at the expense of investment in efforts to study pre-2020 ME/CFS, which may still be driven by distinct biology.

Thus, it is important to both study IACCs separately and to study their overlap. Although symptomatic overlap suggests that some of the same molecular, biochemical, or physiological pathways are affected in these syndromes, mixing study participants whose underlying disruption differs could make a complex, highly variable syndrome even more heterogeneous (*36*). The ultimate goal of much of this research, finding molecular biomarkers, will be made more difficult when multiple IACCs are combined. Although breaking down silos across the IACCs will undoubtedly accelerate progress, studying all IACCs as one condition is premature, because at least some mechanisms for each condition may prove to be unique. Until we know more about the underlying causes of the persistent symptoms of IACCs, we cannot be certain that a treatment for one will be beneficial for another (Fig. 1).

While recognizing the risks of considering all IACCs to be a single disease, we must also acknowledge that shared mechanisms are not only possible but, in our opinion, likely (*34*). Beyond the role of an inciting infection, there appear to be many notable mechanistic similarities among these syndromes. For example, the identification of coagulation disturbances in individuals with Long Covid has been accompanied by similar observations in ME/CFS, although to a lesser degree (*37*, *38*). Chronic inflammation and immune dysfunction are common themes in nearly every IACC (*1*). To truly

investigate similarities and differences between IACCs, direct comparisons must be made in the same study, using the same assays. Incorporating prepandemic ME/CFS cohorts into the studies that will be funded by NIH's ongoing RECOVER initiative, for example, would reveal which mechanisms are shared and whether some elements of pathophysiology differ.

Reactivation of latent human herpesviruses, such as EBV and HHV-6, has been associated with many of these syndromes (*19*, *39*). Although it is unclear whether they are directly responsible for these conditions on their own, herpesviruses could interact through indirect mechanisms by altering a person's susceptibility to another virus, the known ability of these viruses to persist, or more direct mechanisms in the setting of loss of herpesvirus control. Recently, a large study of college students demonstrated that among a subset of those who experienced new mononucleosis and later met criteria for ME/CFS, different metabolite and cytokine profiles could be identified before their illness (*40*, *41*). These studies also revealed that GI symptoms preceding EBV infection, which potentially could be due to a preceding exposure to another pathogen, were strongly associated with the development of ME/CFS. Herpesvirus reactivation has often been seen after other viral infections, physical trauma, or toxic environmental exposures (*42*). Whether herpesviruses play a causative role in failure to recover from COVID-19 requires further exploration.

## ANTIGEN PERSISTENCE AS A DRIVER OF IACCS

By definition, every IACC was caused by an inciting infection. Many of these are assumed to be transient, begging the question: How might exposure to an infection for days to weeks lead to lifelong disability? Theoretically, an acute transient infection could cause irreversible tissue damage or might induce immunologic mayhem, leading to loss of regulation and autoimmunity. Molecular mimicry between the infection and a host protein could also lead to autoimmunity. Recent evidence linking virus burden during acute SARS-CoV-2 infection to Long Covid is generally supportive of these possible mechanisms (*43*–*45*). If true, more aggressive management with antimicrobials during acute infections could reduce the risk of IACCs.

It is easier, however, to build a case for an infection causing a chronic condition if the pathogen persists indefinitely. Whereas the persistence of DNA viruses is well understood, infections with RNA viruses are generally thought to be transient. However, there is evidence that RNA and protein from these viruses can persist beyond the acute phase of illness (*46*). The evidence that SARS-CoV-2 protein or nucleic acid persists indefinitely in some people continues to grow (*10*, *47*–*49*). We and others have consistently found spike protein or RNA in plasma for months or even years after the acute infection (*10*, *49*). It is even easier to find protein and nucleic acid in tissues (*10*, *47*, *50*). Whether this reflects ongoing cycles of viral replication and spread is not known, but the finding that virus can evolve over months in immunocompromised people provides proof that this is at least possible (*51*). To rule out false positives, we recently performed a large survey for the SARS-CoV-2 spike protein in samples from before the pandemic and in samples from people who were weeks to months out after a well-documented SARS-CoV-2 infection. In samples that were collected before SARS-CoV-2 vaccines became available and before re-exposures and re-infections became common, we found a higher proportion of antigen detection in the post-COVID group for up to a year after infection (*49*). Although there are still many caveats, we feel that this can no longer be summarily ignored simply because it does not fit an existing paradigm. Importantly, although the evidence that SARS-CoV-2 can persist is now nearly definitive (*10*, *47*–*50*), the links between persistent virus and Long Covid are weaker. Efforts to make this connection remain one of the most important parts of the research agenda.

Although the pathogen associated with a preceding illness was not identified in most cases of ME/CFS, enteroviruses are most commonly implicated (*2*–*4*, *52*). Enterovirus capsid protein and RNA were found in a large proportion of stomach biopsies from patients with ME/CFS (*53*). Although screening for viruses in easily accessible biospecimens (e.g., blood and saliva) from pre-2020 ME/CFS samples has been negative (*54*), recent experience with SARS-CoV-2 suggests that ultrasensitive methods (e.g., single-molecule assays, peptide arrays, and bacteriophage display) and the use of tissue samples will be needed to definitively assess the possible presence of persistent antigens (*4*). For example, studies that have identified post-acute SARS-CoV-2 antigen have shown that its detection is inconsistent (*49*, *55*) and that detection in tissue might be more reliable (*56*).

## THE EMERGING CLINICAL TRIALS AGENDA AND EXPERIMENTAL MEDICINE FOR IACCS

Unfortunately, there is not yet a solid animal model for studying IACCs; it remains to be seen whether development of such a model is even possible given the subjective nature of these conditions. For this reason, the best scientific strategy for defining the mechanism of each IACC may be to conduct prospective clinical trials in which well-understood interventions in highly characterized participants are used to disrupt specific pathways of interest. We hence advocate for the financing of an experimental medicine agenda that is designed both to uncover mechanisms and identify potential interventions that might prove to be curative in Long Covid, ME/CFS, chronic Lyme disease, and other IACCs. For ME/CFS, the NIH has recently sponsored production of a roadmap to guide research that will help identify promising interventions to be tested in clinical trials (*57*).

The first order of business should be to determine whether pathogen persistence is required for the development and maintenance of disease. Notably, randomized clinical trials of antibiotics targeting *Borrelia burgdorferi* (presumably the cause of chronic Lyme disease) demonstrated promising results in some but not all studies (*58*, *59*); use of these agents remains controversial in clinical care. Uncontrolled case series of small-molecule antivirals have suggested some promise in Long Covid (*60*, *61*), and randomized studies of nirmatrelvir/ritonavir (Paxlovid) in people with established Long Covid are ongoing. This includes a large phase 2/3 study sponsored by RECOVER (VITAL, NCT05595369). Similar studies might be considered in other IACCs, but the lack of effective antimicrobials for most of the inciting pathogens makes such studies impossible to conduct. Biologically, there are also challenges. These approaches require active replication of the pathogen and the administration of an antimicrobial agent that penetrates relevant reservoirs/tissues in a sustained manner. Active replication may not be required, however, because instead it could be that persistent "debris" from the pathogen is inducing a harmful host response. An approach we favor is to target an exposed antigen with pathogen-specific monoclonal antibodies, which have

**Box 1. Cross-cutting research priorities across the spectrum of IACCs.**

**Epidemiology**

Develop clear case definitions for each IACC for use in clinical care and research, recognizing that these will not be the same.

Develop population-based surveillance programs designed to characterize the global incidence and prevalence of each IACC, with an additional focus on low- and middle-income settings in which these conditions are incompletely described.

Enhance population-based surveillance programs in the US and similar countries to better understand health care disparities in IACCs in the settings in which they have most commonly been reported to date.

Improve efforts to understand IACCs in children and adolescents.

Support rigorous, prospective natural history studies of IACCs, including comparative natural history studies.

**Pathobiology**

Definitively determine whether pathogen persistence is the central mechanism for each IACC.

Implement prospective, biologically oriented, linked cohorts designed to conduct cross-disciplinary studies of IACCs.

Study proposed biological pathways, including but not limited to pathogen persistence, inflammation, coagulation, autoimmunity, and herpesvirus reactivation, across multiple IACCs to identify both shared and unique pathways.

Determine why biological sex is a consistent predictor for the incidence of IACCs.

Determine the role of age across the lifespan, focusing on why IACC epidemiology appears to differ in the pediatric versus adult populations.

Determine the impact of IACCs and the pathogens associated with IACCs on maternal-fetal health.

**Diagnostics**

Develop new technologies for the study of IACCs, especially high-throughput methodologies to assess the presence and timing of prior infections.

Develop methods that can determine the identity of the IACC that is causing an individual's symptoms.

Develop measures to monitor the clinical status of those experiencing an IACC over time.

Develop measures for use in clinical trials to assess efficacy of treatments, being mindful that limitations experienced by some individuals with IACCs may preclude participation in certain assessments.

**Therapeutics and clinical care**

Invest in proof-of-concept experimental medicine trials to probe biological pathways for specific IACCs.

Develop antimicrobial drugs that can treat acute or chronic infections that result in IACCs.

Determine the role of acute treatment of the inciting pathogen in preventing each IACC.

Develop vaccines or other prophylactic measures to prevent infections with pathogens that lead to IACCs.

Invest in the rigorous assessment of the role of rehabilitation medicine, appropriate physical therapy, and other disease management approaches within and across the IACCs, being mindful that such treatments may be unhelpful or even harmful in some individuals.

Study different care approaches to IACCs, including monitoring, pharmacologic, rehabilitation, behavioral, complementary medicine, and social support components of care, to define optimal models for these conditions, with a focus on patient-centered, holistic care.

Continue investment in centers of excellence for the care of patients with IACCs, linking care with research.

**Resources**

Enhance the role of community advocates and those with lived experience spanning all arenas related to IACCs, from study of basic mechanisms, to implementation of therapies, to public policy.

Provide a unified set of validated, broadly accessible, centrally housed instruments for use in IACC research.

Implement and promote a series of straightforward diagnostic codes to better understand the burden of IACCs using medical records data.

Maintain infrastructure to rapidly implement epidemiologic investigation of IACC outbreaks, including rapid implementation of biospecimen banking.

Support infrastructure for people who are homebound because of an IACC to be able to participate in research and receive clinical care.

Develop well-characterized IACC cohorts and biorepositories that can be used for cross-IACC research.

Provide resources for national or international meetings to bring IACC researchers together.

Provide funding mechanisms to support IACC-focused multidisciplinary research and cross-IACC research efforts.

the capacity through the Fc receptor to induce a potent host immune response, thus potentially leading to removal of a stubborn reservoir. Monoclonal antibodies have favorable pharmacokinetic profiles that make sustained administration of the antimicrobial more feasible. Case report data suggest potential benefit (*62*). Two of us are now conducting a proof-of-concept study of an anti–SARS-CoV-2 monoclonal antibody in people with Long Covid (NCT05877508); more studies of this approach are needed. If anti–SARS-CoV-2 drugs can restore to health even a subset of individuals suffering from Long Covid, investigating whether a persistent virus underlies prepandemic ME/CFS becomes even more urgent, given that development of a treatment could potentially end decades of suffering for many patients.

Given the consistent finding of immune dysregulation in both ME/CFS and Long Covid, we also believe that immune-modifying strategies should be a priority. For Long Covid, the NIH is funding a large randomized clinical trial of a JAK/STAT inhibitor (baricitinib, NCT05858515), which has the potential to modify several pathways implicated in the condition. Funding to support early, proof-of-concept studies targeting other aspects of the immune system (e.g., the innate immune system), as well as coagulation abnormalities, autoimmunity, and co-infections such as EBV, should also be a priority. Eventually, trials of combinations of therapeutics targeting both upstream biology (e.g., virus persistence and immune dysfunction) and downstream pathophysiology (e.g., autonomic dysfunction and endothelial damage) may be needed (*11*).

## CONCLUSIONS

Despite waning public concern about COVID-19, Long Covid has remained squarely in the spotlight. The field is moving rapidly, with studies posted or published on a near-weekly basis. Still, new funding opportunities and investment in clinical infrastructure to provide care to patients are limited. Industry engagement also remains limited and will likely remain so until progress on defining the mechanism is made. Progress in the management of HIV was only made because of a sustained, decades-long, commitment by the NIH and others. We welcome the recent news that there will be ongoing investment in RECOVER (*63*), but to meet the scope and scale of the

challenge of IACCs will require coordinated, sustained funding beyond this one program. In the absence of such a long-term deep commitment, our current progress in the understanding of Long Covid (and all IACCs) remains under threat (*7*). Still, there is at present optimism that the energy around Long Covid will translate into breakthroughs for other IACCs. The key questions and research strategies that need to be pursued now are similar across the IACCs (Box 1). With deep engagement from those most directly affected by these conditions, now is the time to bring all the IACC stakeholders together, even as the deep dives into mechanisms and treatments remain condition specific.

The late Paul Cheney, one of the two physicians in Incline Village who presided over its famous outbreak, was quoted as saying that "bench researchers are going to take us out of Osler's web" (*5*). He saw the web as a "kind of diffuse feeling that this disease is real, even though we can't find the objective measures" (*5*). Today, many underlying objective abnormalities have been identified in both ME/CFS and Long Covid (*34*). More importantly, the scientific community now has far more tools at its disposal, some that could not even have been imagined 40 years ago. Using these tools to study the full spectrum of IACCs should clear away the web of disbelief and denial, identify those objective measures, and finally provide the knowledge that can lead to beneficial treatments for long-suffering people with IACCs.

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**Acknowledgments:** We acknowledge the patients, providers, researchers, and advocates who have contributed to efforts to understand IACCs. We would like to thank J. Maya for assistance in conceptualizing the figure. **Author contributions:** All authors contributed to the conceptualization, initial writing, and editing of the manuscript. **Funding:** M.J.P. is supported on K23AI157875; the UCSF Long-term Impact of Infection with Novel Coronavirus research program is supported by the PolyBio Research Foundation, with additional support from NIH/NIAID R01AI141003, NIH/NIAID R01AI158013, and NIH/NINDS R01NS136197. The referenced clinical trial (NCT05877508) is supported by the Patient-Led Research Collaborative with additional support from Aerium Therapeutics and the PolyBio Research Foundation. M.R.H. is director of the Cornell NIH ME/CFS Center funded by NIH/NIAID 9U54AI178855. **Conflicts of interest:** M.J.P. has received consulting fees from Gilead Sciences, AstraZeneca, BioVie, and Apellis Pharmaceuticals and research support from Aerium Therapeutics and Shionogi, outside the submitted work. M.R.H. is a member of the scientific advisory boards of the Open Medicine Foundation, Solve CFS/ME, the WE&ME Foundation, and Simmaron Research. S.G.D. reports consulting for Enanta Pharmaceuticals, BioVie, and Pfizer and reports research support from Aerium Therapeutics outside the submitted work.

10.1126/scitranslmed.ado2101

LONG COVID

# Sex differences in postacute infection syndromes

**Julio Silva[1] and Akiko Iwasaki[1,2,3]***

**Postacute infection syndromes like Long Covid disproportionately affect females, differing in prevalence, symptoms, and potential causes from males. This Viewpoint highlights these sex differences, gaps in current understanding, and the critical need for sex-based research.**

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## INTRODUCTION

As the fourth year of the coronavirus disease 2019 (COVID-19) pandemic unfolds, Long Covid has crystallized as a formidable public health challenge. Long Covid afflicts more than 65 million individuals globally with a wide spectrum of symptoms involving multiple organ systems (*1*). Long Covid and other postacute infection syndromes (PAISs) disproportionately affect females. Therefore, understanding PAISs and their link to autoimmunity requires sex-specific research approaches (*2*).

The pathophysiology of PAISs, particularly Long Covid, involves multiple investigated hypotheses with supporting evidence including an ongoing viral reservoir, autoimmunity, latent herpesvirus reactivation, microbiota dysregulation, vascular damage, immune dysregulation, complement activation, and chronic inflammation (*1*, *3*, *4*). Unlike the acute phase of viral diseases, which is more severe in males (*5*, *6*), PAISs are more common and severe in females (*2*, *7*, *8*). The broad and variable symptoms of these syndromes, coupled with a lack of definitive biomarkers, render them diagnoses of exclusion. This raises the critical question of how biological sex, underlying pathologies, and immune responses influence PAIS symptoms and progression.

This Viewpoint explores how sex-related hormonal and chromosomal differences influence PAISs and autoimmune diseases. We also examine current information and gaps preventing better understanding that may, in turn, lead to novel and effective therapies. We focus on the role of biological sex as it pertains to those characteristics of an individual, including reproductive organs, physiology, chromosomal differences, and hormones. It is important to note, however, that gender, a term that broadly encompasses the identity and expression of an individual across various societal roles, undoubtedly plays an important role in the manifestation of diseases, including Long Covid and other forms of PAISs (*9*).

## SEX DIFFERENCES IN RISK FOR PAIS AND AUTOIMMUNITY

Sex differences in PAIS, as seen with autoimmune diseases, show females being more frequently and severely affected than males. Prevalence for Long Covid shows as many as two in three patients are female; for other PAISs, such as myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) and Long Lyme disease, as many as four in five patients are female (*2*, *7*). Females also have a greater burden of symptoms and severity of illness overall (*10*, *11*). Although some PAIS symptoms are as common in males as they are in females [e.g., fatigue, poor sleep, and postexertional malaise (PEM)], others show a sex predominance (e.g., sexual dysfunction for Long Covid in males; neurocognitive problems, pain, hair loss, and dermatological symptoms for Long Covid in females) (*12*). This suggests that PAISs affect the sexes differently, but the mechanisms remain unclear. Different preexisting factors may lead to PAIS development in each sex, or divergent immune responses from the same initial factor may lead to distinct symptoms.

Like PAISs, autoimmune diseases disproportionately affect females, although this depends on disease and age (*13*). Systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) occur in females four times more than in males. SLE peaks between puberty and premenopause (15 to 44 years of age), whereas SS peaks at perimenopausal age (greater than 40 years of age). By contrast, type 1 diabetes (T1D) occurs in males as often as in females but starts in early childhood and early adolescence, before sex hormones peak. Autoimmune diseases may occur simultaneously; some autoimmune diseases, such as celiac disease, SS, SLE, and Hashimoto's thyroiditis, are themselves risk factors for developing others (*13*). Preexisting autoimmune diseases are also risk factors for developing PAISs, including Long Covid, ME/CFS, and Long Lyme disease (*10*, *14*). These shared risks suggest overlapping mechanisms. Hence, studying PAISs in those who have preexisting autoimmune diseases can offer insights into their immunological links and vice versa.

Notably, transgender individuals also face a disproportionate rate of Long Covid, at about two times that of cisgender males (*8*). Transgender individuals also appear to be at higher risk for developing autoimmune diseases, including multiple sclerosis (MS) and SLE (*15*). There is a general paucity of knowledge in our understanding of PAIS and autoimmune disease prevalence within transgender individuals, making it difficult to determine the contribution and interplay among sex, hormone therapies, and gender-based disparities as contributors to disease manifestation.

## SEX DIFFERENCES IN IMMUNE SIGNATURES IN PAIS AND AUTOIMMUNE DISEASES

### Immune signatures in autoimmunity

Autoimmune disease immune signatures show strong sex differences (*16*). Females exhibit more pronounced microRNA and Toll-like receptor (TLR) gene activation on the X chromosome, crucial for type I interferon (IFN-I) signaling and SLE flare-ups (*9*). Increased interleukin-6 (IL-6) production and T follicular helper cells in female rodent models of SLE and rheumatoid arthritis (RA) lead to higher autoantibody titers, correlating with severe disease like organ damage in SLE and relapses in MS (*9*). In experimental autoimmune encephalomyelitis, a

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mouse model of MS, female mice exhibit stronger T helper 1–mediated responses, with higher IFN-γ production; in contrast, male mice develop higher natural killer (NK) cell responses and have increased expression of immunomodulatory signals such as IL-10 and transforming growth factor–β (TGF-β), limiting autoreactive T cell responses (*13*).

### Immune signatures in PAIS

Our study showed female-specific immune responses in Long Covid, characterized by heightened lymphocyte activation (characterized by T cells secreting IFN-γ, IL-2, IL-4, and IL-6), increased T cell exhaustion, and reduced resting naïve $CD4^+$ and $CD8^+$ T cells (*11*, *17*). Immunoglobulin G (IgG) titers against lytic Epstein-Barr virus (EBV) antigens were predominantly elevated in females with Long Covid, suggesting recent reactivation of EBV. These features are potentially driven by reduced testosterone and cortisol concentrations and elevated growth hormone concentrations in females with Long Covid compared with non–Long Covid controls. Males with Long Covid had more NK cells and TGF-β production but fewer monocytes and dendritic cells. Both sexes with lower testosterone concentrations exhibited greater adaptive immune activation and symptom severity (*11*). These findings show sex-biased immunological differences akin to those in autoimmune diseases, suggesting overlapping mechanisms. Elevated autoantibodies against various tissues have been associated with Long Covid and were causally linked to symptoms (*18*, *19*). Sex-stratified analyses are needed to determine whether autoantibody associations in Long Covid are stronger in females than in males, as in other PAISs and autoimmune diseases.

Research into sex differences in immune signatures across various PAISs, such as ME/CFS and Long Lyme disease, reveals patterns similar to those in Long Covid and autoimmune diseases. Females with ME/CFS show dysregulated transcription in naïve T cells linked to severe symptoms like PEM (*20*). Another study reported an increase in circulating naïve $CD8^+$ T cells in females with ME/CFS, whereas males with ME/CFS showed increased CXCR5 expression on $CD8^+$ T cells in cerebrospinal fluid (*21*). Males display heightened NK cell responses, higher IL-10 production, and reduced T cell activation transcriptional signatures. A comprehensive study showed that only 2% of immune and molecular markers in ME/CFS overlapped between sexes (*21*). In Lyme disease, females often show sustained high chemokine (C-C motif) ligand 19 (CCL19) expression, associated with Long Lyme disease (*22*). These studies highlight the substantial impact of sex differences on immune response and disease severity in other PAISs.

### Latent virus reactivation and transcriptional activation of endogenous retroviruses

Latent virus reactivation has been shown to be a key correlate of PAISs and autoimmunity. Viruses like endogenous retroviruses (ERVs) and herpesviruses may be transcriptionally elevated or reactivated, respectively, at any stage of PAISs or autoimmune diseases (*1*, *2*, *11*). Females show higher antibody reactivity to lytic proteins from these latent viruses both before and after PAIS development (*11*, *23*). For instance, female patients with ME/CFS have higher IgM titers against HHV-6 (human herpesvirus 6) and IgG titers against human ERV-K (*23*). Females with ME/CFS also show elevated transcriptional pathways in B cells associated with EBV reactivation, which were associated with higher symptom severity. Similar patterns have been observed in autoimmune diseases. After infectious mononucleosis (caused by EBV primary infection), females who develop MS do so more quickly than age-matched males, especially when infection occurs in late adolescence (*24*). Thus, herpesvirus reactivation rate and immune responses to herpesviruses and HERV are associated with PAIS and autoimmune disease development and are enriched in females.

## SEX DIFFERENCES IN THE BIOLOGY OF PAIS AND AUTOIMMUNITY

The immune systems of females and males exhibit substantial differences (*9, 25*), primarily influenced by sex hormones regulated by the hypothalamic-pituitary-adrenal and gonadal (HPAG) axis and sex chromosomes. These hormonal and chromosomal differences shape the immunological baselines and modulate immune responses to microbial threats. In both PAISs and autoimmune diseases, distinct sex-based patterns exist in HPAG axis dysregulation, immune signatures, latent virus reactivation, and sex chromosome biology. Recurrent themes in sex-based immune responses in PAISs and autoimmune diseases suggest that these differences stem from inherent immunological baselines of each sex. An exhaustive review of baseline immunological sex differences is beyond the scope of this Viewpoint and can be found elsewhere (*9*, *16*, *25*). Here, we focus on differences pertinent to the understanding of sex-related pathological states of PAISs and autoimmune diseases.

### X chromosome dosing and Xist

Sex chromosomes, particularly through X-chromosome gene dosage, substantially affect immune system function and are closely linked to autoimmune diseases (*25*). Key immune genes, such as *TLR7*, *TLR8*, *IRAK1*, *FOXP3*, and *CD40L*, reside on the X chromosome, influencing various immune responses. Individuals with at least two X chromosomes undergo X-chromosomal inactivation mediated by the long noncoding RNA (lcRNA) Xist and other RNA binding proteins (*25*). However, this silencing is incomplete, contributing to higher gene dosage and increased prevalence of autoimmune diseases such as SLE and SS in females.

The components of the Xist ribonucleoprotein (RNP) complex can serve as sources for innate immune stimuli and autoantigens. Female patients with autoimmune diseases can have substantial titers of autoantibodies against Xist RNP components. In a mouse model of pristane-induced lupus, male mice ectopically expressing nonsilencing Xist RNP formed autoantibodies and had more severe multiorgan pathologies than wild-type male mice (*26*). Xist RNP expression in male mice reprogrammed T and B cell populations and chromatin states, making them more closely resemble female mice. In addition to Xist, X-chromosomal dosing has been observed in humans. For example, individuals with Klinefelter's (XXY) or with polysomy X have an increased susceptibility to the development of autoimmune diseases, including SLE, SS, and others. There is a strong interconnection between sex hormones like testosterone, X-chromosomal dosing, and autoimmunity. For example, females with Turner's syndrome (XO) still develop autoimmune diseases at a higher rate. In addition, males with Klinefelter's exhibit lower testosterone than their XY male counterparts, suggesting a strong role of both hormones and X-chromosome dosing in autoimmune diseases (*9*). Given the female bias in autoimmunity and the connection of incomplete X-chromosomal inactivation with autoimmune diseases (*26*), it is worth investigating whether incomplete X-chromosomal inactivation and Xist may contribute to PAIS pathology.

### Baseline sex hormone–mediated differences in immune signatures

Sex hormones fundamentally shape immune responses. Largely because of sex hormone concentrations in healthy adults, T cell, B cell, and NK cell counts differ across sexes. Lower testosterone is chiefly responsible for higher T and B cell counts in females, whereas androgens increase NK cell counts and immune regulatory cytokines like TGF-β and IL-10, dampening adaptive immune responses in males (*9*, *27*). Lower androgen concentrations also result in elevation of post-vaccination antibody titers against viruses like measles, mumps, rubella, influenza, and severe acute respiratory syndrome coronavirus 2 in females (*9*).

These hormonal differences shape immunological baselines and response dynamics in male and female immune systems. For example, castrated male mice vaccinated against malaria produce more antibodies than noncastrated or testosterone-treated counterparts (*28*). Androgen availability and receptor expression inhibit effector $CD8^+$ T cell proliferation, self-renewal, and IFN-γ production, crucial for effective responses against cancer cells (*29*). This may explain why males show poorer responses to some cancer immunotherapies. Importantly, the immunomodulatory effects of androgens have been shown to protect against autoimmunity (*25*, *27*). This protection is evident against the development of autoantibodies and T cell–mediated responses in males with autoimmune diseases and may explain why prepubertal autoimmune diseases such as T1D are shared more equally across sexes than those that manifest after puberty. Categorizing androgens solely as immune inhibitors, however, oversimplifies their wider role in shaping the immune baseline, influencing gene expression differences in male and female immune cells at the tissue level (*30*).

One recent study has also shed light on the immunological impacts of gender-affirming hormone therapy (GAHT) in transgender individuals (*31*). This longitudinal study investigated immune system adaptations in transgender men undergoing testosterone therapy (TT) and found that testosterone modulates a cross-regulated axis between IFN-I and tumor necrosis factor (TNF) (*31*). Specifically, male dose concentrations of testosterone attenuated IFN-I responses in plasmacytoid dendritic cells and monocytes while potentiating monocyte responses leading to increased TNF, IL-6, and IL-15 production (*31*). Testosterone also potentiated IFN-γ responses in NK cells. This dynamic regulation of immunity by sex hormones provides critical insights into how GAHT may influence the susceptibility and manifestation of PAISs and autoimmune diseases in transgender individuals.

Estradiol and progesterone also substantially affect the immune system. Estradiol can either silence or stimulate immune functions, whereas progesterone modulates NK cell activity, inflammatory signals, and antiviral responses (*9*, *25*). In most females who develop autoimmune diseases such as SLE and SS, symptoms usually begin after puberty but before menopause, suggesting an important role for these sex hormones (*9*).

These baseline immune differences create distinct advantages and disadvantages. Females benefit from a robust adaptive cell-mediated and humoral response, offering protection against acute infections with lower severity, but are at higher risk of developing PAISs and autoimmune diseases. Conversely, males have an immune response that provides better protection against many autoimmune conditions and may protect against PAISs but have an increased risk of developing severe acute infections. These differences are particularly evident when examining dysregulated sex hormones and HPAG dynamics typical of these conditions.

### Sex hormone dysregulation

In PAISs and autoimmune diseases, sex hormones influence the age of disease onset, immunological patterns, and symptoms in both sexes. PAISs are most prevalent among females of premenopausal and perimenopausal age (*7*, *8*), with the sex difference in incidence most pronounced in this group, although females are at higher risk across all ages. In children, boys develop Long Covid nearly as frequently as girls, especially before puberty (*8*, *30*). Pregnancy appears to protect against certain ME/CFS symptoms, and a common feature of Long Covid and other PAISs is menstrual cycle disruption (*11*, *32*). These findings suggest a critical role for hormones in PAIS pathogenesis.

Testosterone concentrations are key predictors of sex-based immune differences in individuals with Long Covid. Lower testosterone is linked to female-predominant adaptive T cell and humoral immune signatures in both females and males (*11*). Males with lower testosterone also show higher rates of ME/CFS, Long Lyme disease, and autoimmune diseases, with up to 40% of all males with MS having lower testosterone levels (*9*, *13*, *15*, *16*, *27*). Conversely, higher testosterone concentrations correlate with male-predominant immune signatures, fewer symptoms, less organ involvement, reduced PEM, and lower neurocognitive impact associated with Long Covid, regardless of sex. Our recent findings indicate that testosterone strongly negatively associates with herpesvirus reactivation in both sexes, suggesting a role for testosterone in maintaining viral latency (*11*).

Androgens may reduce proinflammatory signatures in PAISs and autoimmune diseases through direct and indirect pathways, including up-regulation of TGF-β and IL-10 (*9*, *16*, *25*). This limits T cell, B cell, and NK cell effector functions, such as production of TNF-α, IFN-γ, and granzyme, as well as perforin release and development of autoantibodies. However, higher doses of testosterone may paradoxically also stimulate a pro-TNF inflammatory pathway. It remains unclear whether androgens may reduce autoantibody production in PAIS. Androgens might also limit the self-renewal and proliferation of B and T cells, including autoreactive cells, and enhance regulatory T cell ($T_{reg}$) development (*13*, *31*). Single-nucleotide polymorphism studies and mass spectrometry metabolomics studies indicate lower androgen steroid pathway factors as a defining feature in females with ME/CFS, suggesting a potential role for androgens as diagnostics or therapeutics in PAISs (*32*–*34*).

Estradiol and progesterone are also implicated in PAISs and autoimmune diseases (*9*, *35*). Males with Long Covid have lower estradiol concentrations than controls, and females with ME/CFS show dysregulation of these hormones (*32*). In addition, pregnancy, which affects estradiol and progesterone concentrations, has been shown to either benefit or worsen symptoms in both PAISs and autoimmune diseases (*9*). These differences may stem from the dual role of estrogen hormones in activating adaptive immune responses and inducing regulatory factors like IL-10 (*9*). Further research is needed to clarify their role in PAIS autoimmunity.

### HPAG axis dysregulation

HPAG dysregulation is a key factor in the pathologies of PAISs and autoimmune diseases (*32*, *35*, *36*). Signs in Long Covid, such as lower cortisol and sex hormones and altered pituitary hormones like growth hormone, suggest that a systemic endocrine imbalance contributes to sex-specific symptoms. These irregularities are primarily linked to hypothalamic and pituitary dysfunction. For instance,

sexual dysfunction in males with Long Covid is associated with lower testosterone because of damage to gonadotropin-releasing hormone cells in the hypothalamus (*36*). HPAG dysfunction affects the production and signaling of sex and other steroid hormones like cortisol, influencing immune responses and viral reactivation in a sex-specific manner (*11*, *33*, *34*). In ME/CFS, hormones such as cortisol, testosterone, estradiol, luteinizing hormone, and follicle-stimulating hormone are implicated, with studies showing disruptions in steroid pathways and circadian rhythms (*32*, *34*). Similar dysregulation is observed in SLE and MS. It remains unclear whether HPAG dysregulation is a primary cause or a consequence of PAIS pathogenesis (*35*). A better understanding of HPAG axis dysregulation could provide insights into PAISs and potential treatment opportunities.

### Hormone therapy

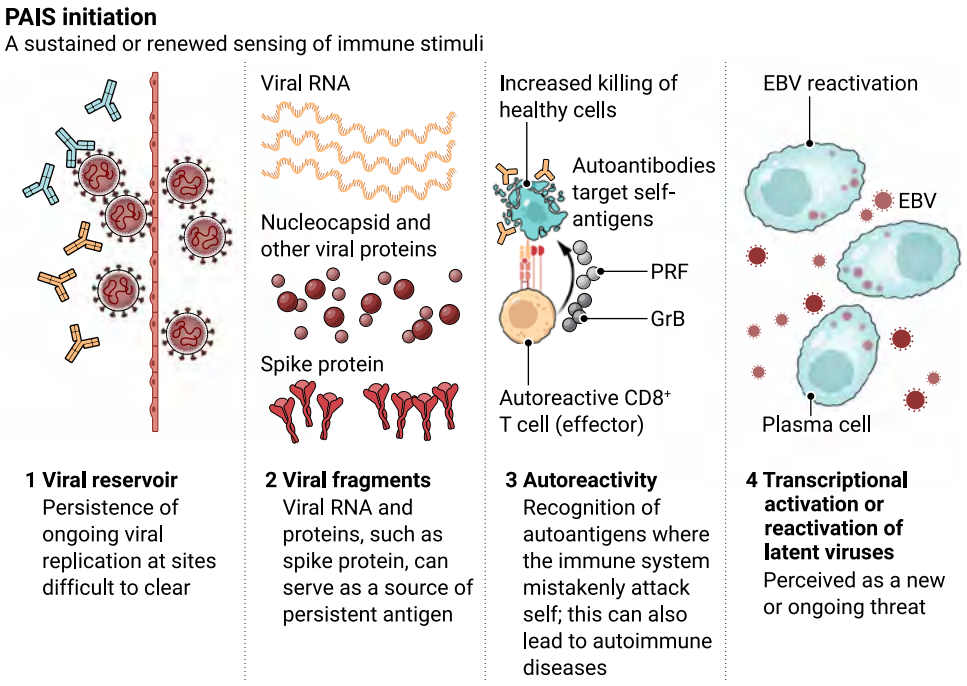
TT and other androgen therapies have led to symptom improvements in SLE and MS in both sexes in multiple mouse and a few human studies (*35*, *37*). For instance, a pilot study with 10 male patients with MS showed that TT (100 mg daily) improved cognitive performance and slowed brain atrophy over a year, although it did not reduce lesions (*32*). Because of the small number of studies and participants, definitive conclusions on testosterone's therapeutic potential are lacking. Similar testosterone-associated symptom profiles in PAISs suggest that TT might help individuals with PAISs, although this remains unexplored (*11*, *36*). Estradiol treatment has been shown to benefit some patients with ME/CFS and MS but has also been shown to exacerbate SLE symptoms (*32*). More research and larger clinical trials are needed to clarify the effect of hormone therapy for PAISs and autoimmunity.

## HYPOTHETICAL FRAMEWORK FOR UNDERSTANDING PAIS IN FEMALES AND MALES

To develop PAISs, we propose that the following two criteria must be met. The first applies to both sexes, whereas the second differs by sex. The first is sustained or renewed sensing of continued immune stimuli due to persistent viral reservoir, persistent viral antigen (proteins, RNA, or both), persistent autoantigen, or reactivation of latent viruses sensed as a recall threat (Fig. 1). The second criterion to be met is the presence of exacerbating factors that lower the threshold for tolerance and regulation, driving inflammatory immune responses (Fig. 2). In females, these include greater adaptive immune responses to threats by X chromosome–encoded factors, greater TLR and innate responses through increased X-chromosome dosage, elevated Xist leading to persistent immune complexes as autoantigens, and lower androgen concentrations, which drive a pro–adaptive immune inflammatory state. In XY males, exacerbating factors include HPAG axis disruption and lower testosterone, which may drive a higher proinflammatory adaptive immune response. Elevated NK cells may also induce lytic and inflammatory conditions in males.

## CLINICAL IMPLICATIONS, GAPS, AND STEPS TOWARD TRANSLATION

The overlap between PAISs and autoimmunity highlights shared mechanisms and differing manifestations across sexes, underscoring the need for sex-disaggregated analyses and tailored diagnostic tools and therapies. Distinct immune signatures in PAISs suggest that testosterone concentrations could serve as both a diagnostic biomarker and a therapeutic target for a particular endotype of PAIS. Preliminary studies indicate that even clinically normal variations in testosterone can markedly influence immune responses and symptoms, suggesting its potential utility in managing PAISs. However, major gaps remain in our understanding,




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**Fig. 1. PAIS initiation requires a sustained or renewed sensing of continued immune stimuli.** The factors required to initiate or continue PAISs include (1) a persistent viral reservoir, (2) persistent viral antigen (proteins, RNA, or both), (3) persistent autoantigen, or (4) reactivation of latent viruses that the immune system senses as a recall threat (which may, in turn, lead to persisting viral antigens or autoantigens). PRF, perforin; GrB, granzyme B; EBV, Epstein-Barr virus.

**Sex-specific differences**
Conditions that can exacerbate or dampen the immune response, affecting tolerance and regulation of inflammatory processes

Females Xa Xi

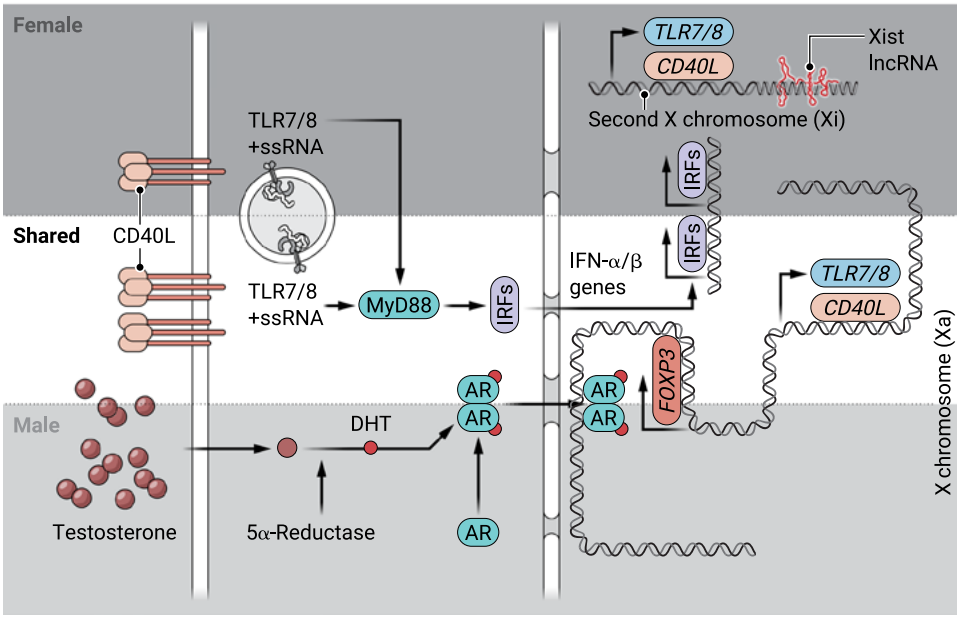
**Higher basal X-chromosome adaptive immune mediators**
(e.g., CD40L expression) Leads to increased amplification of effector T cell functions

**Higher basal X-chromosome TLR dose**
Higher maximal capacity for type 1 IFN induction

**XIST-mediated inflammation**
Formation of XIST RNP complexes can lead to immune responses against them (1.3)

**Lower testosterone or androgen concentrations**
Drives an adaptive and inflammatory state

↑ $CD4^+$ T cells, $CD8^+$ T cells, B cells ↑ IFN-γ, antibodies ↓ NK cells, $T_{reg}$ cells ↓ IL-10, TGF-β




Males X Y

**Lower basal X-chromosome adaptive immune mediators**
(e.g., CD40L expression) Leads to lower amplification of effector T cell functions

**Lower basal X-chromosome TLR dose**
Lower maximal capacity for type 1 IFN induction

**No XIST expression**
No risk of immune response to XIST RNP complexes

**Higher testosterone and androgen concentrations**
Dampens adaptive immune responses and inflammatory state

↓ $CD4^+$ T cells, $CD8^+$ T cells, B cells ↓ IFN-γ, antibodies, IFN-I ↑ NK cells, $T_{reg}$ cells ↑ IL-10, TGF-β, TNF

**Fig. 2. Sexually dimorphic factors can regulate the immune response, affecting tolerance and activation of inflammatory processes.** These factors include greater adaptive immune responses to sensed threats by X-chromosome factors (e.g., CD40L), greater TLR and innate immune responses through increased X-chromosome dosing, incomplete X-chromosomal inactivation, which may lead to immune complexes that persist as autoantigens, and androgen concentrations driving a pro–adaptive immune inflammatory state that increases effector T cell responses, B cell antibody responses, and the likelihood of viral reactivation. Female factors are shown on the top. Male factors are shown on the bottom. Shared factors are shown in the center. ssRNA, single-stranded RNA; IRFs, interferon regulatory factors; DHT, dihydrotestosterone; AR, androgen receptor; MyD88, myeloid differentiation primary response 88; *FOXP3, forkhead box P3* gene.

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particularly in regard to why PAIS rates are higher in females. This disparity may also explain the higher prevalence of autoimmunity in females. Further research is essential, especially studies exploring the roles of the X chromosome and Xist in PAIS development, to elucidate the complex role that biological sex plays under these conditions.

## CONCLUSION AND OUTLOOK

The similarities between PAISs and autoimmunity suggest shared mechanisms. We argue that sustained or renewed sensing of chronic threats, combined with sex-specific factors, initiates PAIS and autoimmunity development. Sex differences, through factors including sex chromosomes and hormones, alter immune responses and shape the operational state of the immune system.

A better understanding of how immune responses that underlie PAISs and autoimmune diseases differ between females and males will inform better-targeted therapies. For example, modifying immune responses through sex-tailored hormone therapy may improve symptom severity and reduce autoimmunity risks. Much work remains to understand how hormones and other factors affect PAISs and autoimmune diseases and translate this knowledge into effective therapies.

We advocate for including diverse study populations and adopting patient-centric approaches to better understand how biological sex affects Long Covid, ME/CFS, Long Lyme disease, other PAISs, and autoimmune diseases. Despite their potential to substantially contribute to our scientific understanding of PAISs and autoimmune diseases, current research has yet to extensively focus on the development of PAISs in transgender individuals, a group disproportionately afflicted with PAISs and one uniquely positioned to illuminate the interplay between biological sex differences and the impact of sex hormones on disease outcomes. Engaging transgender individuals in PAIS research, especially those undergoing GAHT, may continue to reveal how hormonal profiles and chromosomal differences shape the immune system, influencing severity and manifestation. Studying PAISs and autoimmune diseases in pediatric populations could further help decipher how sex hormones, age, and development contribute to disease. Such studies are crucial for advancing our understanding of PAISs and developing more effective therapeutic approaches.

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**Acknowledgments:** We thank S. Borah for editorial assistance. **Funding:** A.I. is an investigator of the Howard Hughes Medical Institute. Our work discussed in this Viewpoint was supported in part by the Else Kröner Fresenius Prize for Medical Research 2023, a grant from the National Institute of Allergy and Infectious Diseases (R01AI157488), and the FDA Office of Women's Health Research Centers of Excellence in Regulatory Science and Innovation (CERSI).
**Competing interests:** A.I. cofounded RIGImmune, Xanadu Bio, and PanV and is a member of the Board of Directors of Roche Holding Ltd. and Genentech. J.S. declares no competing interests.

10.1126/scitranslmed.ado2102

Science Translational Medicine

LONG COVID

# Symptoms after Lyme disease: What's past is prologue

**Adriana Marques***

**Protracted fatigue and other symptoms can occur after Lyme disease and other infections, with numerous possible drivers. Studies on posttreatment Lyme disease have been inconclusive, with no confirmed biomarker emerging. Prolonged antibiotic therapy provides no benefit. Thus, a holistic approach toward understanding and treating this complex disease is necessary.**

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## INTRODUCTION

The question of why some patients fail to recover from an infection and continue to have unexplained fatigue and other symptoms has been long debated. Possible mechanisms, not necessarily exclusive, include factors associated with host immune response (immune dysregulation and autoimmunity), antigen or pathogen persistence, misdiagnosis, comorbidities, and psychosocial influences. The cause of persistent symptoms after antibiotic treatment of Lyme disease and the concept of chronic Lyme disease (CLD) continue to be highly controversial topics, originating from historical, social, and biological aspects of the disease (*1*–*3*).

Lyme disease, or Lyme borreliosis, is caused by spirochetes of the genus *Borreliella* (also named *Borrelia burgdorferi* sensu lato complex or Lyme borrelia) that are transmitted by the bite of infected ticks of the *Ixodes ricinus* complex. First recognized in the United States in the mid-1970s, Lyme disease is the most common vector-borne illness in the temperate zones of the Northern Hemisphere (*2*). The geographic range and number of cases continue to increase because of environmental factors, including climate change (*4*). Lyme disease in North America is caused by infection with *B. burgdorferi*, with a few cases by *B. mayonii*. *Ixodes scapularis* (the black-legged or deer tick) is the main vector of the infection, and *Peromyscus leucopus* (the white-footed mouse) is the key reservoir for *B. burgdorferi* in the United States. Lyme disease in Europe is predominantly caused by *B. afzelii* and *B. garinii*, followed by *B. bavariensis* and *B. burgdorferi* (*5*).

For clinical purposes, Lyme disease is divided into early localized, early disseminated, and late stages, but manifestations can overlap and appear independently. Lyme disease usually starts with erythema migrans, a distinctive skin lesion at the site of the tick bite. Systemic symptoms without erythema migrans or preceding erythema migrans can also be initial manifestations. Within days or weeks from initial infection, *Borreliella* can disseminate and establish infection at distant sites. Early disseminated manifestations can include multiple erythema migrans lesions, early Lyme neuroborreliosis, and Lyme carditis. Lyme arthritis, a later manifestation, usually occurs 6 months after the initial erythema migrans. Borrelial lymphocytoma and acrodermatitis chronica atrophicans are manifestations seen in infections acquired primarily in Europe.

Clinical variations occur depending on the infecting *Borreliella* species and virulence within the same species (*5*). Lyme arthritis is mostly associated with *B. burgdorferi*, whereas *B. garinii* and *B. bavariensis* are associated with Lyme neuroborreliosis. Borrelial lymphocytoma and acrodermatitis chronica atrophicans are primarily caused by *B. afzelii*. Systemic symptoms, fever, and multiple skin lesions are more common in patients with erythema migrans caused by *B. burgdorferi* infection acquired in the United States, compared with *B. afzelii* and *B. garinii*. Infection with *B. burgdorferi* strains classified as ribosomal spacer type (RST) 1, particularly subtype A of the outer surface protein C (OspC), is more likely to disseminate hematogenously, cause more severe early disease, and carry a higher risk of postinfectious Lyme arthritis (*6*). The mechanisms underlying these differences are not understood but relate to gene dosage and allelic variation in surface-expressed lipoproteins that help *Borreliella* evade host immune response and facilitate binding and invasion of host tissues (*7*).

The lack of a sensitive direct diagnostic test substantially contributes to the controversy surrounding Lyme disease. Direct detection of *Borreliella* in clinical samples is difficult for most clinical presentations because of the low bacterial load in human disease and preferential localization of *Borreliella* in tissues. Current laboratory tests are based on detection of antibody responses in serum. Serodiagnostics approaches work relatively well if used as recommended, and the modified two-tier testing algorithm has improved sensitivity in early Lyme disease. Still, because of the window period, patients early in their illness can have negative test results. These patients should receive treatment on the basis of clinical diagnosis. Interpretation of serologic results can be confusing to health care providers and patients, and false-positive results can occur, particularly with immunoglobulin M (IgM)–based tests. Importantly, current assays do not distinguish between active and inactive infection and cannot be used to monitor response to therapy. This complicates serodiagnosis of reinfections and can cause confusion for patients. The use of nonvalidated Lyme diagnostic tests is problematic and not recommended (*8*).

## SYMPTOMS AFTER TREATMENT OF LYME DISEASE

Most patients fully recover after treatment with recommended antibiotic regimens. Patients with later manifestations can take longer to recover, and some damage can be nonreversible, as seen in patients with residual weakness due to facial nerve palsy. Treatment failure, with objective signs of disease after treatment, is uncommon. Postinfectious Lyme arthritis, or antibiotic-refractory

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Lyme arthritis, is a complication that occurs in about 10% of patients with Lyme arthritis (*9*). These patients continue to have inflammatory proliferative synovitis despite 2 to 3 months of antibiotic therapy and are treated with disease-modifying antirheumatic drugs. Host genetic factors, such as Toll-like receptor 1 (*TLR1*) gene polymorphism and certain human leukocyte antigen (*HLA*)–*DR* alleles, together with *B. burgdorferi* virulence factors, such as infection with *B. burgdorferi* RST1 OspC type A strains, are associated with postinfectious Lyme arthritis (*6*). Infection results in an excessive proinflammatory immune response that continues into the postinfectious period with high amounts of interferon-γ (IFN-γ), increased T helper 1 ($T_H1$) and $T_H17$ immune responses, and autoreactive T and B cells. *B. burgdorferi* peptidoglycan has been detected in synovial fluid from patients with postinfectious Lyme arthritis and likely has a role in maintaining inflammation (*10*).

Posttreatment Lyme disease symptoms or syndrome (PTLDS) resembles other postinfectious fatigue syndromes. These individuals have unexplained persistent or relapsing nonspecific symptoms starting within 6 months after treatment of a documented episode of Lyme disease (*1*). The most common complaints are fatigue, difficulties with concentration and memory, myalgias, arthralgias, depression, anxiety, paresthesias, and sleep problems. In comparison with another post infectious condition, Long Covid, shortness of breath, changes in smell or taste, palpitations, chest pain, and cough are not reported in PTLDS. The prevalence of subjective complaints 6 to 12 months after treatment ranges from 0 to 27% in prospective studies of patients with erythema migrans (*11*–*37*). This is similar to the estimated prevalence of Long Covid, ranging from 0.3 to 2% (*38*), 6.6 to 10.3% (*39*), and 12.7% (*40*), and to the prevalence of persistent fatigue or pain after other infections.

## POSSIBLE CAUSES OF PTLDS

Risk factors associated with PTLDS resemble other postinfectious syndromes, providing clues to components contributing to persistent symptoms. Factors related to the infection include more severe disease, as marked by presentation with multiple erythema migrans or disseminated manifestations, higher symptom severity at the start of treatment, the presence of tingling or an abnormal skin sensation at the initial visit, higher bacterial load in skin, and antibiotic treatment delay. Other risk factors include older age, female sex, presence of comorbidities, poor social and physical functioning, history of stressful events, more negative perceptions of illness, preexisting anxiety or depression, and higher anxiety and depression scores at the start of antibiotic treatment (*26*, *27*, *30*, *32*, *41*–*44*). Most likely, several factors contribute to predisposing and perpetuating these symptoms in an individual patient (Fig. 1).

### Convalescence, misdiagnosis, and other conditions

Most patients continue to improve after treatment for Lyme disease, with fewer patients reporting symptoms over time. This is an expected convalescent period, as seen with other infections, and an important point to emphasize and reassure worried patients. Moreover, symptoms and signs due to other conditions can be wrongly attributed to Lyme disease, with an expected poorer response to treatment. For example, patients with a definite diagnosis of Lyme neuroborreliosis have better outcomes after treatment when compared with possible cases (*45*). Similarly, nonspecific symptoms can be caused by preexisting or new conditions unrelated to Lyme disease. The presence of comorbidities is associated with long-term symptoms and lower quality of life scores in Lyme disease (*42*), and it can be difficult to attribute or exclude a nonspecific symptom to a specific condition. Of note, coinfections with other tick-borne pathogens are rare in PTLDS (*41*, *46*–*48*).

### Common factors

Nonspecific symptoms like fatigue, chronic pain, and sleep disturbances are common in the general population. Prospective studies examining patients with Lyme disease and controls showed more than 20% prevalence of these symptoms in controls. Five prospective studies showed a similar or lower prevalence of symptoms in patients with early Lyme disease (*22*, *33*, *34*, *36*, *37*), although two studies showed higher prevalence of symptoms in patients (*27*, *35*) when compared with that in controls. The LymeProspect cohort study, conducted in The Netherlands, is particularly interesting (*35*). This study compared the prevalence of persistent fatigue, cognitive impairment, and pain between cohorts of 1135 patients with Lyme disease, 2405 individuals with a tick bite, and a general population cohort of 4000 individuals. The prevalence of symptoms at the 1-year follow-up was 27.2% in the Lyme disease cohort, 23.3% in the tick bite cohort, and 21.2% in the general population cohort. An analysis of baseline factors associated with persistent symptoms at 1 year found that the main predictors were similar between patients and controls (*41*). These predictors included poorer physical and social functioning; higher depression and anxiety scores; more negative illness perceptions; comorbidities; and more severe fatigue, cognitive impairment, and pain. The cognitive-behavioral responses to symptoms also contributed to the prediction of symptom persistence. A Lyme disease diagnosis was only moderately associated with the presence of symptoms. The importance of an individual's positive or negative expectancies on symptom course and treatment outcome has also been shown in the Persistent Lyme Empiric Antibiotic Study Europe trial (*49*). Predictors of symptom improvement at both the end of treatment and at follow-up were higher pretreatment functioning, higher pretreatment expectancy to improve, and thinking that one had received antibiotics at the end of therapy (*50*). Previous exposure to traumatic life events was associated with a higher risk of PTLDS in patients with early Lyme disease (*27*). These results align with studies researching common predisposing and associated factors of complex, multidimensional symptoms like fatigue and pain. Knowledge about these factors can help identify patients at risk and develop interventions to prevent, reduce, and help manage these symptoms. How to control for these variables and integrate them into therapeutic research is a challenging topic.

### Pathogen and host factors

The local early immune response and the interplay with bacterial virulence factors are important in controlling the infection and potentially affect host response, disease manifestations, and outcome. Patients with erythema migrans who had a positive culture by skin biopsy were less likely to fully recover than patients with a negative culture (*30*, *51*). Skin biopsy culture positivity is associated with bacterial load and *Borreliella* burden in the skin (*30*, *52*). Prior work showed that the bacterial load was nearly three times higher in RST1 compared with that in RST2 and RST3 *B. burgdorferi* (*52*) and that RST1 strains are more frequently associated with disseminated infection and with postinfectious Lyme arthritis. There is no evidence linking differences in *Borreliella* virulence and PTLDS, but this likely reflects difficulties

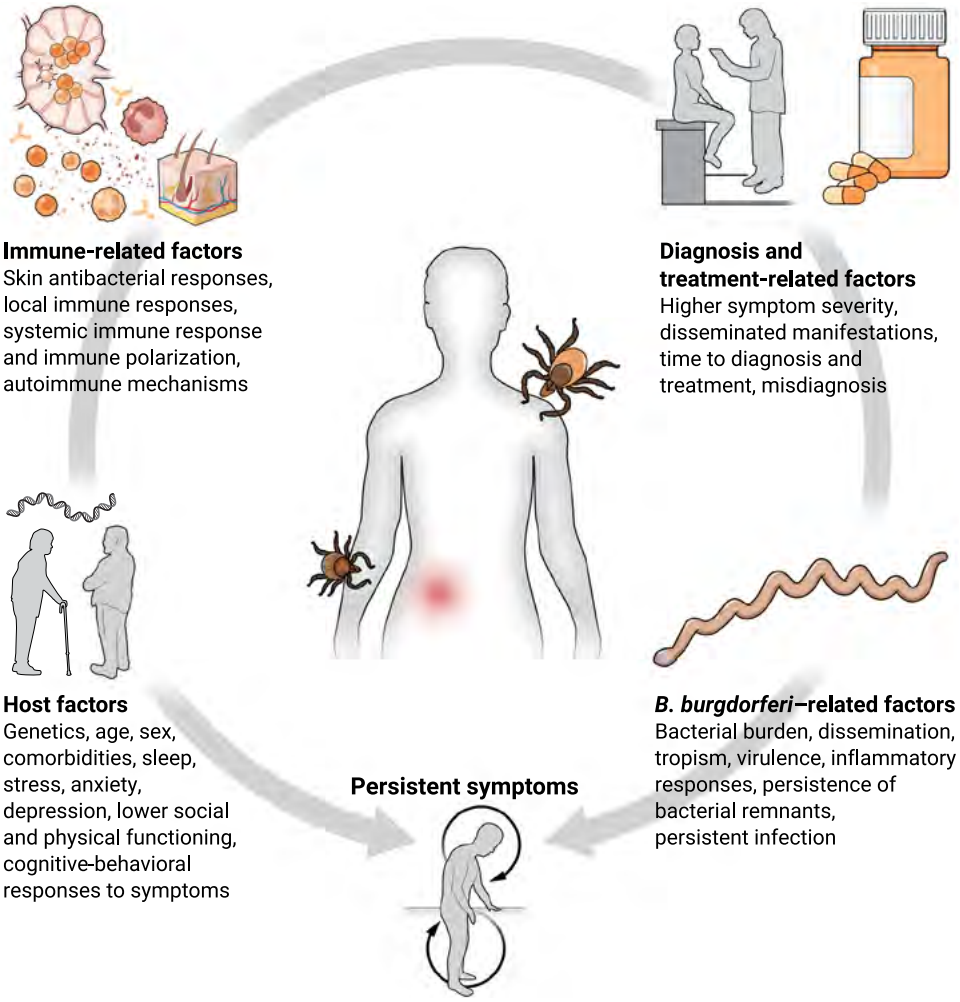



**Fig. 1. Potential factors contributing to PTLDS.** Several factors may interact to predispose to, or protect against, PTLDS. These include factors contributing to the effectiveness of the host immune response against the pathogen and potentially dysregulated immune responses. These may be intrinsic (genetics, age, sex, and comorbidities) and extrinsic (psychological stress, disturbed sleep, and poor health behaviors) to the host. Host factors interact with *B. burgdorferi*–related factors, which influence pathogen burden, inflammatory responses, bacterial dissemination, and tissue tropism. Delays in diagnosis and the start of antibiotic treatment influence pathogen control, disease presentation, and host responses. Misdiagnosis and other conditions can cause delays in seeking adequate care. Importantly, how an individual experiences illness will play a role in recovery.

in obtaining direct pathogen information from most patients.

Host genetic factors influence the skin antibacterial response to *B. burgdorferi*, as well as innate and adaptive immune responses against the bacteria, and are associated with susceptibility to Lyme disease, more severe presentations, and maladaptive immune responses (*6*, *53*–*55*). A lower expression of IFN-γ in erythema migrans skin biopsies was associated with PTLDS, supporting the hypothesis that a local $T_H1$ response is important early in the infection (*56*). Patients who returned to health had a higher number of plasmablasts at the time of diagnosis than individuals who developed PTLDS, suggesting that initial activation of B cells may influence recovery, but larger studies are needed (*57*). A missense variant at the gene encoding for secretoglobin family 1D member 2 (*SCGB1D2*), a protein expressed primarily in the skin and sweat glands, was linked to an increased susceptibility to Lyme disease, possibly because of less efficient inhibition of the bacteria in the skin (*53*). Increased Lyme disease susceptibility was also found to be associated with the rs1061632 major allele, possibly related to up-regulation of the Akt–mammalian target of rapamycin (mTOR) pathway and interleukin-10 (IL-10) responses, along with reduced specific antibody production, which may have a negative impact on bacterial clearance (*55*). A study using samples from the LymeProspect cohort (*35*) showed that cytokine responses were associated with age and sex, as well as Lyme disease manifestation and antibody production. Moreover, the study identified 34 genetic loci associated with cytokine responses, including *TLR1-6-10*, *IL6*, *IL10*, and *Klotho*, a gene encoding a protein linked to aging and age-related illnesses (*58*). The *TLR1-6-10* locus had the strongest association, with fine mapping linking to rs5743618-A. Also part of the LymeProspect study, IL-1β, IL-6, IL-10, and IL-1 receptor antagonist stimulation responses ex vivo, as well as 13 single-nucleotide polymorphisms in seven genes, were examined as predictors of persistent symptoms. Lower IL-10 concentrations at

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6 weeks were associated with persistent symptoms in the primary analysis, and polymorphisms in mannose binding lectin and IL-1β were associated with attribution of persistent symptoms to Lyme disease in a subgroup analysis (*41*).

Older age is a risk factor for PTLDS, and younger patients with early Lyme disease were more likely to have a complete response (*26*). A prior study demonstrated that age impaired IFN-γ and IL-22 production in peripheral mononuclear cells stimulated with *B. burgdorferi* (*59*). Older patients have more comorbidities, which can confound symptom assessment and may increase vulnerability. Certain comorbidities may interfere with host response to Lyme borrelia and predispose to persistence of symptoms. For example, in one murine study, diet-induced obesity led to more severe pathology and inhibition of innate immune response to *B. burgdorferi* (*60*); in another mouse study, the authors found that poor sleep delayed arthritis resolution, possibly by altering host immune response (*61*). Poor and insufficient sleep correlates with fatigue, pain, anxiety, and cognitive complaints in humans, and it is an important generic factor implicated in predisposing and perpetuating fatigue due to many conditions. It also can be targeted for intervention (*62*–*64*). Female sex is a risk factor for PTLDS, as it is for most postinfectious fatigue syndromes as well as fatigue due to other conditions. Potential contributory factors are multifold and may relate to both psychosocial and biological factors (*65*).

## Autoimmunity and immune dysregulation in PTLDS

The role of infection-induced autoimmunity and immune dysregulation in PTLDS has been investigated, but no reproducible marker has been identified. The concentrations of C-reactive protein, a proinflammatory marker, were shown to be higher in patients with PTLDS than those in recovered patients, suggesting that inflammation could play a role in some individuals (*66*). Other studies found that individuals with PTLDS had higher titers of antibodies to neural proteins (*67*, *68*). Antibody titers against endothelial cell growth factor were higher in one study (*69*) but not confirmed in another (*70*). Median concentrations of chemokine (C-C motif) ligand 19 (CCL19) were found to remain elevated at posttreatment visits in individuals with PTLDS, possibly reflecting an ongoing immune-driven process (*71*). These findings were not replicated in a European study, which instead found an association with persistent symptoms and higher IL-23 concentrations, suggesting the possibility that a $T_H17$ response could play a role in persistent symptoms (*69*). A $T_H17$-associated immune response has been linked to autoantibody production in postinfectious Lyme arthritis (*72*, *73*) and slower recovery in Lyme neuroborreliosis (*74*, *75*). Two studies have implicated IFN-α in PTLDS, with a recent study in patients with Lyme neuroborreliosis showing higher IFN-α concentrations in sera at the initial and follow-up visits in individuals with PTLDS when compared with those in recovered individuals and that IFN-α concentrations correlated with severity of symptoms (*76*, *77*). This is an interesting link because fatigue, myalgia, and cognitive dysfunction are known side effects of IFN-α treatment.

## Transcriptomic responses to Lyme disease

The longitudinal transcriptomic response in blood has been investigated in a series of studies, but again no clear markers have been found to inform PTLDS pathogenesis. The first study found that transcriptomic changes due to the acute infection (erythema migrans) did not return to baseline at 6 months after treatment, but few differences were observed between recovered patients and patients with persistent symptoms (*78*). These sustained transcriptomic changes were not found in a study of patients with early disseminated infection (*79*). A distinct gene expression signature was seen during acute disease and early convalescence but resolved by 6 months after treatment. This study identified a 20-gene classifier that could distinguish patients with acute Lyme disease from controls. A third study demonstrated that gene expression profiles of patients with erythema migrans clustered separately from controls, with most patients in clusters 0 and 1 and most controls in cluster 2 (*80*). Patients in cluster 0 had more symptomatic acute disease, whereas patients in clusters 1 and 2 were more likely to have received initial antibiotic therapy. However, machine learning classifiers could not clearly distinguish which patients would develop PTLDS. Most cases stayed separate from controls over time (6 and 12 months), but a batch effect is possible in this study. The same group investigated the transcriptional profile of patients with PTLDS compared with that of patients with acute Lyme disease and controls using the data from the previous study (*81*). A classifier with 35 genes specific to Lyme disease could distinguish individuals with acute Lyme disease, individuals with PTLDS, and healthy controls. Because the groups were processed separately, the division between PTLDS and the other groups may be explained by batch effects. Recently, a 31-gene Lyme disease classifier was developed using transcriptomic data from patients with early Lyme disease, controls, and patients with other infections (*82*). The potential of this classifier to evaluate patients with the diagnosis of Lyme disease and symptoms after therapy requires further study. Of note, there is no overlap when comparing the genes selected for each of the three classifiers distinguishing acute Lyme disease from controls.

## Persistence of *B. burgdorferi* or its remnants

One possibility is that remnants of *B. burgdorferi* could persist in tissues after antibiotic treatment, driving a persistent host response in some patients. *B. burgdorferi* peptidoglycan was found in synovial fluid of patients with postinfectious Lyme arthritis (*10*), but no studies in PTLDS have been performed to date. The questions of whether there are residual live *Borreliella* after antibiotic treatment and whether that could be driving PTLDS have been a major concern. In animal studies, low amounts of *B. burgdorferi* DNA and mRNA can be detected in tissues for months after antibiotic treatment, and *B. burgdorferi* DNA has been detected by xenodiagnosis (the use of a vector to detect the presence of an organism) in some studies (*83*–*89*). However, the bacteria could not be recovered by culture. A clinical study using xenodiagnosis with laboratory-reared *I. scapularis* larval ticks was safe but resulted in a positive test in only 1 of 17 patients with PTLDS or high titers of antibodies to *B. burgdorferi* C6 peptide (*90*). The importance of detecting *B. burgdorferi*'s DNA and whether it represents viable *B. burgdorferi* or remnants of the bacteria is a point of ongoing discussion (*91*).

There have been five randomized, placebo-controlled, double-blind clinical trials addressing the question of whether additional antibiotic treatment benefits patients with PTLDS or symptoms attributed to Lyme disease. Regimens tested against matching intravenous and oral placebos included ceftriaxone for 30 days followed by doxycycline for 60 days (*46*), ceftriaxone for 28 days (*92*), and ceftriaxone for 10 weeks (*47*). One study had an open-label period of ceftriaxone for

2 weeks, followed by random assignment of patients to 12 weeks of oral doxycycline, clarithromycin-hydroxychloroquine, or placebo (*49*). The results of these trials showed that prolonged antibiotic treatment had no lasting benefit while having potential serious risks. The studies also showed a placebo effect and that the intensity of symptoms varied over time. Additional studies of antibiotic treatment of PTLDS will require discovery of biomarkers that identify distinct subgroups and provide a strong rationale for new interventional trials. In addition, therapeutic interventional studies in PTLDS must have a double-blind, randomized controlled design.

## A NOTE ABOUT CLD

The controversy surrounding Lyme disease dates to the early investigations of the disease, with differences about how Lyme disease is defined (and therefore diagnosed and treated), leading to the CLD debate that continues to this day. CLD is a contentious term, with no clear diagnostic criteria. For example, one definition is a "multisystem illness with a wide range of symptoms or signs that are either continuously or intermittently present for a minimum of 6 months." CLD has evolved into a "multiple chronic infectious disease syndrome," and patients are diagnosed with numerous infections and poorly defined conditions. Patients are often charged high fees for diagnosis and care and may spend money on unproven tests and treatments that range from harmless to dangerous. Other medical conditions may be delayed in diagnosis and treatment or can be missed altogether. Patients with PTLDS are a small subset of this large and heterogeneous group. Most patients with CLD have other conditions or are patients with persistent physical symptoms (*93*–*102*). Because of the lack of a consensus definition for CLD or on the myriad of treatments used, it is not possible to make any conclusive statements about the condition(s), but there is a critical need for high-quality research to better understand and help this diverse group of individuals.

## CONCLUSION

Unfortunately, the questions about the underlying causes of PTLDS remain mostly unanswered. Studies have been inconclusive with findings that have not been replicated. The reasons are multifold, including heterogeneous populations, differences in methodology, and multiple tested variables combined with a small number of patients suffering from PTLDS in prospective studies. Currently, there are no confirmed biomarkers to help differentiate between subgroups of patients, which are necessary to help personalize care for patients suffering from prolonged symptoms. A holistic approach encompassing all aspects of the illness experience can help reduce and manage complex symptoms, and associated factors should not be overlooked. Lyme disease is a challenging area for research, and there is a critical need for more investment in fundamental, translational, and clinical research. A supported clinical network to facilitate high-quality clinical studies in all different stages of Lyme disease can help bring evidence-based answers to these open questions and improve the care of patients. Moreover, a comprehensive multidimensional model addressing common factors predisposing individuals to persistent symptoms after infections and other triggers, combining expertise across different fields, can lead to new ways to prevent, treat, and manage these conditions.

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**Acknowledgments**
**Funding:** Funding for this study was provided by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US government.
**Competing interests:** A.M. has a patent (US 8,926,989) issued and is an unpaid scientific adviser to the Global Lyme Alliance and to the American Lyme Disease Foundation.

10.1126/scitranslmed.ado2103

LONG COVID

# Animal models of Long Covid: A hit-and-run disease

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**The severe acute respiratory syndrome coronavirus 2 (SARS-CoV 2) pandemic has caused more than 7 million deaths globally. Despite the presence of infection- and vaccine-induced immunity, SARS-CoV-2 infections remain a major global health concern because of the emergence of SARS-CoV-2 variants that can cause severe acute coronavirus disease 2019 (COVID-19) or enhance Long Covid disease phenotypes. About 5 to 10% of SARS-CoV-2–infected individuals develop Long Covid, which, similar to acute COVID 19, often affects the lung. However, Long Covid can also affect other peripheral organs, especially the brain. The causal relationships between acute disease phenotypes, long-term symptoms, and involvement of multiple organ systems remain elusive, and animal model systems mimicking both acute and post-acute phases are imperative. Here, we review the current state of Long Covid animal models, including current and possible future applications.**

## INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic has caused more than 770 million infections and more than 7 million documented deaths globally, reconfirming the dreadful consequences of sudden zoonotic virus emergence events on human morbidity and mortality, as well as on the global economy. The COVID-19 pandemic, coupled with other viral outbreaks, has also blurred the historic distinctions between "acute" and "chronic" disease states, either with or without ongoing virus persistence in immune-privileged sites. Even in the absence of evidence of viral persistence in the case of COVID-19, hit-and-run disease phenotypes have emerged, perhaps associated with dysregulated reparative functions or remodeled immune cell responses that program long-term immunopathological disease outcomes across organs (*1*). These chronic disease features have resulted in unexpected health care challenges for disease control and quality-of-life outcomes in survivors globally, requiring new mitigation efforts. The ongoing COVID-19 pandemic has also demonstrated that preparing for current and possible future infectious disease threats requires thoroughly engaged public-private partnerships to elucidate the mechanisms and host response patterns that drive the transition between acute and long-term chronic disease outcomes and to rapidly realign pandemic preparedness from the current reactive response platform into one that is proactive.

More than 65 million people are predicted to suffer from Long Covid symptoms globally (*2*). Treating these manifestations is challenging because of the presence of preexisting conditions, disease heterogeneity, different clinical care regimens, and case definition ambiguities. Moreover, the treatment regimens needed to demonstrate clinical improvement are often difficult to quantify, challenging treatment performance in more than 110 ongoing Long Covid clinical trials (*3*). Highly refined animal models of human Long Covid represent a high priority requirement for identification of molecular mechanisms and correlates of chronic disease improvement. Here, we discuss the current state and future directions of Long Covid animal models.

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## CORONAVIRUSES CAUSE DISEASE IN MAMMALS

Coronaviruses are a large group of enveloped viruses. Coronaviruses encode a single-stranded positive polarity RNA genome of about 30,000 nucleotides (nt) in length, surrounded by a helical nucleocapsid and a lipid envelope that minimally encodes a 23-kDa membrane (M) glycoprotein, the envelope (E) structural protein, and a large 180-kDa spike glycoprotein (S). Coronaviruses primarily infect mammals and birds, often causing severe acute gastrointestinal or respiratory diseases, although others can cause chronic inflammatory syndromes systemically or in the brain (*4*). Coronaviruses that target the respiratory tract, including sarbecoviruses [e.g., severe acute respiratory syndrome coronavirus (SARS-CoV) and SARS-CoV-2] and merbecoviruses [e.g., Middle East respiratory syndrome coronavirus (MERS)–CoV], often infect epithelial cells in the nose and the conducting airways, as well as cells in the gas exchange region of the lung, which can result in surfactant loss, atelectasis, hypoxia, pneumonia, acute lung injury, and death (*5*). In addition to acute symptoms, coronavirus infections may progress to long-lasting chronic immunopathological diseases in the brain and other organs, even after live virus clearance (*6*).

Four contemporary human coronaviruses (HCoVs)—HCoV-229E, HCoV-OC43, HCoV-HKU1, and HCoV-OC43—are recognized as causing mild disease in most human populations, and long-term persistence of viral RNA has been detected in brain autopsy samples (*7*). More recently, emerging coronaviruses have resulted in severe life-threatening illness with high mortality rates ranging from 1 to 35%, usually with more severe consequences in older adults and immunosuppressed individuals. In the 21st century, two distinct group 2b sarbecoviruses (SARS-CoV 2003 and SARS-CoV-2 2019) and one group 2c merbecovirus (MERS-CoV) have caused outbreaks and pandemics of severe, life-threatening illness in human populations. Zoonotic reservoirs have revealed the presence of numerous group 2b, group 2c, and group 1 coronavirus strains that are also poised for emergence (*8*–*13*), roughly defined as being able to (i) use human receptors, such as angiotensin 1 converting enzyme 2 (ACE2) or dipeptidyl peptidase 4 (DPP4), for docking and entry; (ii) replicate efficiently in primary human cells; and (iii) cause severe disease in humanized mice (*9*, *10*). Given that some serological studies predict about 50,000 introductions of zoonotic sarbecoviruses into human populations in Southeast Asia each year (*14*), the dire global health consequences of emerging coronaviruses cannot be understated.

## LONG COVID IN HUMANS

Although most survivors recover from acute SARS-CoV-2, others develop long-term chronic disease sequelae resulting in clinical symptoms in the brain and lung, manifesting as brain fog, confusion, shortness of breath, cardiac complications, and loss of quality of life for months to years after primary infection. In addition, breakthrough SARS-CoV-2 infections are associated with increased chronic disease risk (*15*). Although typically associated with severe acute infection, mild infections may also progress to chronic outcomes even in young children and healthy adults (*2*). The long-term global health and economic impact of these chronic disease endotypes remains high.

Early during the COVID-19 pandemic, a subset of patients presented with various symptoms that persisted long after the acute phase (post-acute sequelae of COVID-19) (*2*). Over time, survivors and clinicians recognized that some patients reported a plethora of persistent disease symptoms in the brain, lung, kidneys, and other organs. Now, Long Covid is recognized as a multisystemic condition with more than 200 identified symptoms that occur at least 3 months after infection and that may resolve with or without recrudescence or continue for years (*2*, *16*). Symptoms range from shortness of breath, anosmia, chronic fatigue, and cardiovascular conditions to neurological, cognitive, and psychiatric sequelae; severe chronic headaches, confusion, and brain fog; and chronic bowel manifestations and other organ abnormalities (*17*). Repeat infections are frequently associated with either increased disease manifestations or the emergence of Long Covid symptoms in previously healthy individuals (*18*). In addition, breakthrough SARS-CoV-2 infections are associated with increased chronic disease risk (*15*). Symptoms can fluctuate, complicating clear definitions of Long Covid and resulting in a frustrating ordeal for patients as well as medical professionals. A Long Covid disease definition has recently been published by an expert working group for the National Academy of Sciences (*19*).

Among the possible long-term manifestations of SARS-CoV-2, a causal relationship between acute viral respiratory infection and post-acute pulmonary sequelae is most plausible, likely mediated through acute virus replication leading to tissue damage, elevated cytokines, and immune-mediated pathology. It is also clear that, preexisting conditions, age, obesity, sex, and repeat infections may influence the onset, magnitude, duration, and severity of Long Covid phenotypes in patients. Cross-organ communication networks appear to promote, regulate, and influence the magnitude and maintenance of long-term disease outcomes, especially between the lung and central nervous system (CNS) compartments (*20*). Respiratory conditions are a common phenotype in individuals with Long Covid, some of whom develop organizing pneumonia with or without pulmonary fibrosis months to years after acute infection. Females generally experience more severe manifestations in the brain and lung, although cohort variation as a function of sex have also been widely reported (*17*, *21*). African American, Hispanic, and Native American populations exhibit disproportionate burdens of chronic disease after SARS-CoV-2 infection (*22*). Likewise, females and older adults are more prone to suffer chronic disease manifestations (*23*).

Long-term disease phenotypes have also been demonstrated in the kidney, heart, and other organs after infection with SARS-CoV-2 or other coronaviruses (*24*). Survivors from both the 2003 SARS-CoV and 2012 MERS-CoV epidemics had persistent disease phenotypes including fatigue, shortness of breath, reduced quality of life, and cognitive or behavioral disorders (*25*). Because robust acute SARS-CoV and MERS-CoV mouse and hamster models have been reported, opportunities exist to evaluate, compare, and contrast the underlying mechanisms by which merbecovirus infection elicits chronic disease phenotypes in the lung and other organs (*26*–*29*). An unfortunate lack of robust mouse models for contemporary human coronavirus has restricted our understanding of acute and chronic disease phenotypes and should be recognized as a potential important future direction for the field.

## LONG COVID ANIMAL MODELS

Current definitions of Long Covid include evolving phenotypes, because different organ-specific sequelae may present with a spectra of disease manifestations. Because the molecular drivers for Long Covid disease phenotypes in the lung and CNS remain poorly defined in human populations, robust animal models are key for understanding the basis of disease progression and outcomes as well as for testing or developing therapeutics. For the purposes of this Review, acute infection in standard laboratory animals is defined by disease features seen through day 15 and associated with live virus clearance, subacute infection occurs between days 15 and 30, and chronic disease occurs beyond 30 days after viral infection in the absence of detectable live virus. It is recognized that some, but not all, patients (or animal models) may present with low amounts of viral RNA but not live virus, especially in immune privileged sites (*30*, *31*).

### Mouse models

Mice are the most widely used small mammalian model in virus acute and chronic disease research. Their small size; short reproduction time; and the extensive list of related cell biology, genetic, and immunological tools and reagents make the mouse the most versatile and cost-effective mammalian model organism. Despite some profound differences in rodents and humans, in the context of SARS-CoV-2, many disease determinants, genetic susceptibility loci/genes, and phenotypic outcomes are highly reproducible between the two species (*32*–*34*). In addition to a plethora of studies comparing host responses across highly pathogenic respiratory viruses (*35*), common susceptibility loci and prioritized gene candidates exist between mice and humans, allowing for the study of conserved host genes on both acute and chronic disease outcomes (*32*–*34*).

Modeling viral acute and chronic infection outcomes and pathogenesis across species, where host genetic variation and anatomical structure are not fully shared, complicates animal model development that fully phenocopies human outcomes. However, two general strategies with a high degree of success have been used to enhance reproducibility across species: (i) modifying the host receptor to allow for efficient virus docking and entry into murine target cells or (ii) adapting human virus strains to replicate efficiently in murine hosts by serial passage in vivo. For respiratory viruses, humanized mice implanted with human lung or other tissue provide a complementary platform for study (*36*, *37*).

#### *Long Covid lung phenotypes in mice*

To model long-term effects on the lung in mice, various groups have extended experimental time frames past the acute phase (days 1 to 15) through the subacute phase (days 15 to 30) to the chronic phase. In general, models have been developed using (i) adeno-associated virus vectors (AAVs) to deliver coronavirus receptors (*38*), (ii) CRISPR-Cas9 to edit mouse entry receptors (*27*), (iii) human ACE2–overexpressing transgenic mice (*39*, *40*), (iv) spike receptor

binding domain molecular modeling and mutagenesis (*41*), (v) serial passaging in vivo to adapt virus to cause severe pulmonary disease outcomes (*28*, *42*, *43*), and (vi) immunodeficient mice implanted with human lung tissue (human lung–only mice) (*36*, *37*). All of these models demonstrate efficient in vivo replication of SARS-CoV, SARS-CoV-2, and MERS-CoV. Several models have been evaluated for disease phenotypes from day 7 to day 30 after infection, whereas others have demonstrated chronic lung and CNS disease phenotypes between day 30 and day 120 after infection (Fig. 1) (*44*, *45*). In wild-type mice, evidence of long-term viral persistence is lacking after day 30, although at this time, it is likely premature to rule out any possibility for a low amount of persistent viral RNA in immune privileged sites because coronavirus sequences have been known to persist in these sites for years (*46*). In immunosuppressed mice, infectious long-term sarbecovirus persistence has been demonstrated as well (*47*).

One Long Covid mouse model for lung and brain phenotypes relied on a mouse-adapted virus strain (*44*, *45*). To investigate Long Covid pathogenesis, infected mice were followed from acute to clinical recovery phases (day 2 to day 120 after infection). After virus clearance by day 15, pulmonary histological findings identified subpleural lesions composed of collagen, proliferative fibroblasts, and chronic inflammation, including tertiary lymphoid structures. Elevated cytokine concentrations, as well as frequencies of CD4 T cells, B cells, and interstitial macrophages, were prominent in the chronic phase. Longitudinal spatial transcriptional profiling identified global reparative and fibrotic pathways dysregulated in diseased regions, similar to observations in human COVID-19 cases. Populations of alveolar intermediate cells, keratin (Krt)8$^{+}$ alveolar differentiation intermediate (ADI) cells, damage-associated transient progenitor (DATP) cells, or pre–alveolar type 1 (AT1) transitional cell state (PATS) cells accumulated to promote a persistent cycle of dysfunctional airway remodeling, inflammation, and fibrosis. These senescent cells secrete cytokines and other factors that recruited and potentially maintained a proinflammatory and profibrotic environment and triggered fibroblast and myofibroblast activation (*48*). Early intervention with the antiviral molnupiravir reduced fibrotic phenotypes in chronic disease phases, and early antifibrotic agent (nintedanib) intervention modified early, but not late, disease severity, leading to a potential model associated with aberrant reparative processes, epithelial cell remodeling, and prolonged cytokine production that promotes a chronic proinflammatory environment with associated fibrotic remodeling in the lung (*44*) (Figs. 1 and 2). In the brain at 60 days, neuronal damage and increased numbers of ionized calcium-binding adapter molecule (Iba) 1–positive amoeboid microglia persisted in the hippocampus, a region critical for long-term memory (*49*).

Conceptually, this basic model is supported by other mouse models of human disease. For example, human ACE2 transgenic mice, in which ACE2 is delivered

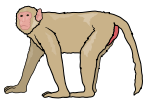
| | Mouse (WT) | Genetically modified mouse | Hamster | Ferret | NHP |
|---|---|---|---|---|---|
| |  |  |  |  |  |
| **Experimental model** | Mouse-adapted SARS-CoV-2, development of ALI and ARDS | WT SARS-CoV-2 clinical isolates | ACE2 protein and expression pattern similar to humans, WT SARS-CoV-2 | Similar lung physiology to humans, replication in upper respiratory tract, respiratory transmission | Similar genetics to humans, shedding |
| **Long Covid** | Pulmonary fibrosis, collagen deposition, hyperproliferation of fibroblasts, chronic inflammation, neuro-inflammation | Pulmonary fibrosis, collagen deposition, hyperproliferation of fibroblasts, chronic inflammation, anosmia | Pulmonary fibrosis, chronic inflammation, cardiovascular condition, neuro-inflammation | Often only minimal to mild pathological changes, transmission model, preclinical testing | Neuro-inflammation, neuropathology, brain hypoxia |
| **Study duration** | 0–120 dpi | 0–15 dpi | 0–31 dpi | 0–22 dpi | 0–265 dpi |
| **Therapeutic interventions** | Model system for acute infection, preclinical testing, molnupiravir and nintedanib tested | None reported | Model system for acute infection, preclinical testing, transmission studies | Model system for acute infection, preclinical testing, transmission studies, molnupiravir and Paxlovid tested | Preclinical evaluation |
| **References** | Dinnon *et* (*44*) Amruta *et* (*45*) Trevino *et* (*54*) | Zheng *et al.* (*40*) | Heydemann *et al.* (*72*) Frere *et al.* (*74*) Serafini *et al.* (*77*) | Au *et al.* (*84*) Lieber *et al.* (*88*) | Huot *et al.* (*92*) |

**Fig. 1. Usage of current SARS-CoV-2 animal models to study Long Covid development.** Mouse, hamster, ferret, and NHP models have been developed to study different aspects of Long Covid and to test efficacy of candidate Long Covid therapeutics. WT, wild type; ALI, acute lung injury; ARDS, acute respiratory distress syndrome; dpi, days post infection.

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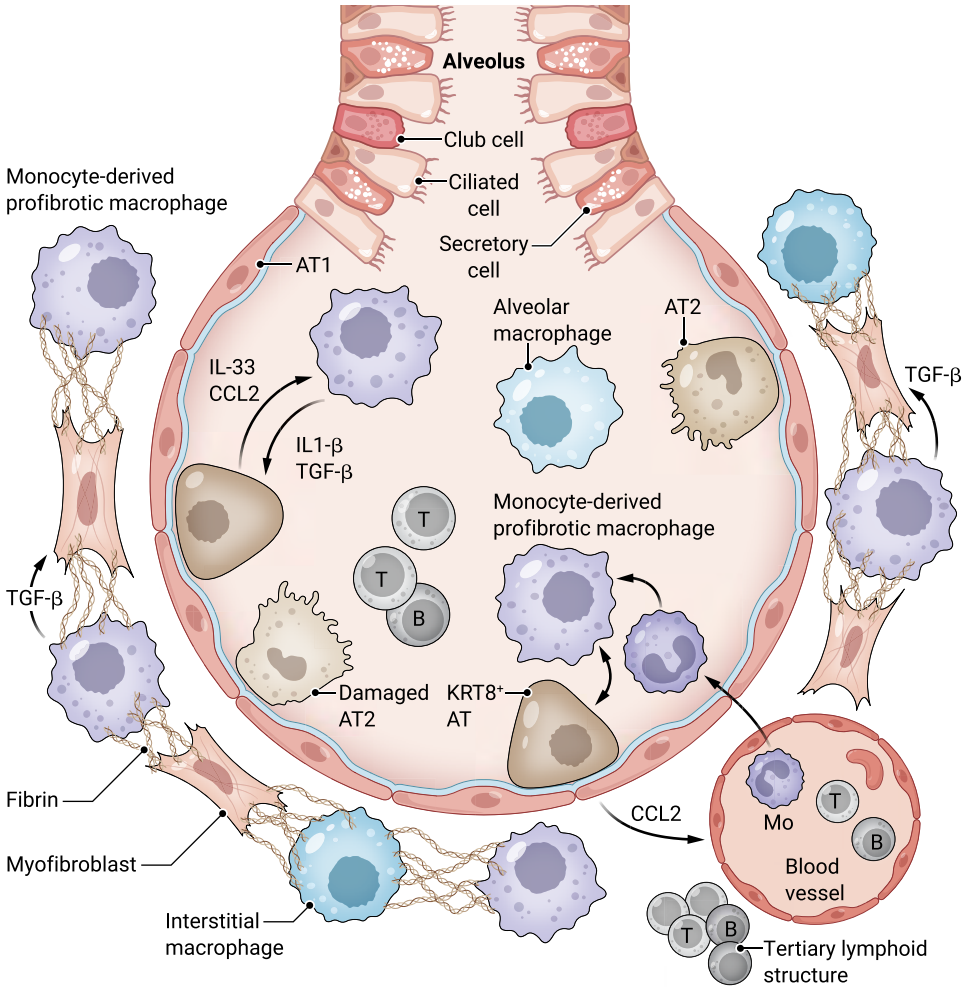



**Fig. 2. Mechanisms of coronavirus infection damage in the lung during Long Covid.** AT2 cells are the primary target of SARS-CoV-2 infection, which causes AT2 cell loss, initiating reparative processes to repair this cellular defect. In the process of recovery, some cells become frozen in AT1 to AT2 transitional states, leading to the expression of KRT8$^{+}$ in AT cells as well as the production of proinflammatory and profibrotic cytokines, such as IL-33, IL-6, CCL2, and TGF-β. These cytokines recruit inflammatory macrophages and monocytes and other immune cells to the site of injury and promote myofibroblast proliferation, collagen deposition, and fibrosis. Mo, monocyte; T, T lymphocyte; B, B lymphocyte; IL, interleukin; CCL, C-C motif chemokine ligand; TGF-β, transforming growth factor β; KRT8, keratin 8.

by AAVs to generate a humanized mouse model, were infected and followed for 28 days. In the lung, there was evidence of fibrosis with prolonged interferon-stimulated gene signatures and inflammatory macrophage responses, which are also responsible for detrimental immune pathologies (*31*). Using an immunosuppressed, transduced human mouse model, macrophage replication was induced by SARS-CoV-2 infection, leading to macrophage enlargement and the formation of lipid-laden cells. The enlarged macrophages displayed a profibrotic and prothrombotic phenotype, and even after virus clearance, macrophage numbers remained elevated when lung fibrosis and thrombi formation were still present (*50*).

### *Neurological Long Covid in mice*

Cognitive impairments—ranging from anosmia, brain fog, and memory loss to anxiety and depression—are often reported in individuals with Long Covid. Mouse models that capture these chronic disease symptoms have been reported, linking infection in the nasal epithelium and lung to cognitive impairments. In the olfactory epithelium, SARS-CoV-2 infection of sustentacular cells induced myeloid cell infiltration, increased proinflammatory cytokine expression, and caused widespread damage to olfactory sensory neurons (OSNs), including olfactory receptor downregulation and altered olfaction-related signal transduction. OSN dysfunction not only reduced the numbers of tyrosine hydrolase–expressing neurons in the olfactory bulb but also caused inflammation and olfactory dysfunction that persisted for weeks after infection in many animals (*51*). Impaired olfaction has been tightly linked to cognitive decline and neurodegeneration in the brain, providing a potential pathway for persistent CNS disease and cognitive disorders after acute virus infection in both mice and humans (*52*, *53*).

In a study that modeled outcomes after mild acute SARS-CoV-2 infection, mice exhibited increased memory loss and cognitive deficits postrecovery that were concordant with blood-brain barrier (BBB) breakdown, increased T cell infiltration of the brain, and microglial activation (*54*). In another report studying severe SARS-CoV-2 infection using a mouse-adapted virus, brain interleukin-6 (IL-6) concentrations were elevated, BBB integrity was reduced, and brain glial fibrillary acidic protein mRNA expression was increased. An increase in perivascular lymphocyte cuffing at 30 days after infection was noted among female BALB/c mice (*45*). Fernández-Carteñeda and colleagues delivered human ACE2 by AAVs to the upper and lower respiratory tracts of mice, then infected the mice with human SARS-CoV-2 isolates (*55*). They observed that cerebrospinal fluid cytokine concentrations and microglial reactivity remained elevated through 49 days after infection. In addition, they found that C-C motif chemokine ligand 11 likely activated hippocampal microglial cells and interfered with neurogenesis-biological parameters that were connected to neurological and behavioral symptoms seen in humans, such as cognitive impairment (*55*). Using a completely different approach, cognitive dysfunction was recorded for 30 to 60 days after infusion of spike glycoprotein into the mouse CNS. Toll-like receptor 4 blockage reversed these symptoms (*56*). Regardless of the approach, a common theme derived from animal model studies is that acute virus infection of the olfactory epithelium, airways, and gas exchange region of the lung can promote deleterious outcomes that directly or indirectly disrupt cognitive function, coupled with immune cell involvement in the maintenance and persistence of these symptoms (Fig. 3). Another study argues that Long Covid is

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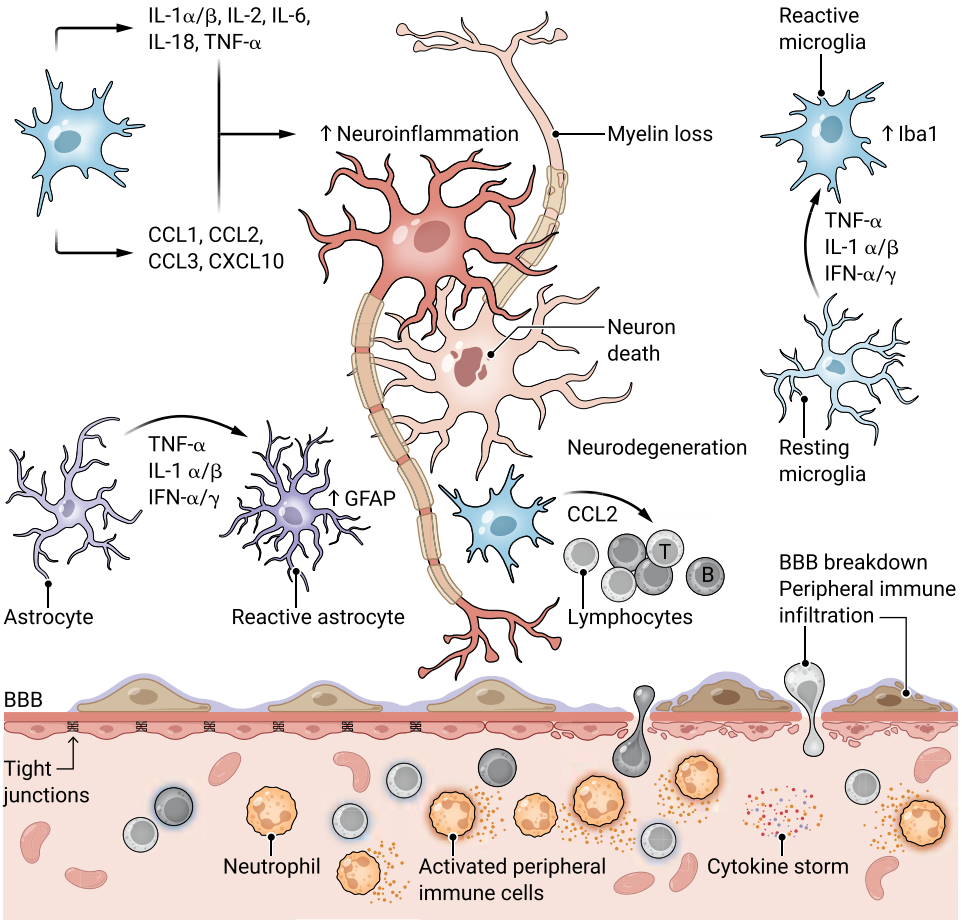



**Fig. 3. Mechanisms of coronavirus infection damage in the brain during Long Covid.** Acute infection causes neuroinflammation and neuropathology in the brain, like microglial and astrocyte activation, which can develop into Long Covid. The neuroinflammatory milieu caused by activated microglia leads to breakdown of the BBB, perivascular cuffing, and infiltration of peripheral immune cells, such as activated T and B lymphocytes, monocytes, and neutrophils. Activated microglia cause myelin and oligodendrocyte loss, neuronal death, and neurodegeneration. CXCL, C-X-C motif chemokine ligand; TNF-α, tumor necrosis factor α; IFN, interferon; GFAP, glial fibrillary acidic protein; Iba1, ionized calcium-binding adaptor molecule 1.

associated with serotonin reduction, which impedes vagus nerve function, hippocampal responses, and memory (*57*).

## Hamster models

The Syrian hamster, otherwise known as the golden hamster (*Mesocricetus auratus*), has been used in a wide variety of research areas, including cancer and infectious disease, in response to a critical need to develop alternative models for studying human disease outside classic rodent models. In the field of infectious diseases, the Syrian hamster model has been well established for studying the pathogenesis of SARS-CoV-2 and developing therapeutics and vaccines. Although the hamster model historically suffered from a lack of key immunological reagents and genetic approaches to manipulate the genome, recent advances have begun to resolve that gap. A strength of the hamster is that airborne transmission models have provided critical insights into the underlying mechanisms that govern spread in populations (*58*, *59*). In addition, the human *ACE2* gene has successfully been introduced into hamsters by gene editing, supporting a potential pathway for the development of human *DPP4* hamster models as an important platform for the investigation of acute and chronic merbecovirus pathogenic and transmission mechanisms (*60*, *61*).

The Syrian hamster model is established as an outstanding model for emerging coronavirus infections, especially SARS-CoV-2 and its variants of concern (VOCs), as well as additional related zoonotic strains (*11*, *58*, *62*–*66*). SARS-CoV-2 uses ACE2 orthologs for entry across species and replicates to high titers in lung and nasal turbinates of Syrian hamsters, with peak replication in lower airways on day 2 after infection and clearance by day 7 after infection (*62*, *67*). Lung pathology is most severe by 7 days after infection and resolves by 14 days after infection (*62*, *67*). In contrast, MERS-CoV (EMC/2012) does not infect and replicate in the Syrian hamster (*68*, *69*), and the 2003 SARS-CoV strain replicates without serious clinical disease (*70*). Animals followed for 21 days after MERS-CoV infection showed no signs of disease and no pathological lesions in the lungs (*71*).

Target cells for SARS-CoV-2 in the lungs are (i) bronchial epithelia cells, (ii) type I and type II alveolar cells, and perhaps (iii) macrophages. Viral replication in the hamster lung was shown to be accompanied by inflammation and pulmonary damage, such as severe lesions, focal infiltration of immune cells resulting in interstitial pneumonia, alveolar septum thickening, pulmonary edema, and alveolar hemorrhage, with disease being most severe between 5 and 7 days after infection (*58*, *62*, *67*, *72*, *73*). Consequently, infection results in a moderate to severe bronchointerstitial pneumonia, characterized by acute inflammation and cell death early during infection, progressing to proliferative interstitial pneumonia with type II pneumocyte hyperplasia by day 7 after challenge, with pulmonary lesions resolving between 10 and 14 days after infection (*62*). The hamster model develops Long Covid–like chronic disease, although most studies have only focused on the subacute phase of infection through or before day 30; therefore, longer-duration studies are needed to demonstrate whether disease is progressive or resolves over time (*72*, *74*).

Heydemann *et al.* (*72*) investigated alveolar regeneration in SARS-CoV-2–infected hamsters using morphological, phenotypical, and transcriptional readouts. Inflammatory lesions with prominent epithelial proliferation were observed during the acute phase of infection, which lasted up to 7 days after infection. On the basis of earlier studies in mice and humans, the authors confirmed the emergence of AT2-ADI-AT1 intermediate cells during the subacute phase up to 14 days after infection and further demonstrated that cytokeratin (CK) $8^+$ ADI cells and multipotent $CK14^+$ airway basal

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cells were both involved in alveolar regeneration. They also showed that the prolonged persistence of ADI cells is associated with the development of fibrosis in infected hamsters (Fig. 2) (*72*). More detailed studies through longer time points will be necessary to determine a common role for intermediate epithelial cells in the initiation and establishment of Long Covid in the lung.

A more systemic evaluation of long-term SARS-CoV-2 infection in hamsters was performed by Frere *et al.* (*74*), who showed that a systemic antiviral and inflammatory response was initiated during the acute phase of infection and was resolved by about 14 days after infection. Despite the similarity of the extent and characteristics of the inflammatory and antiviral response mounted in the lungs and peripheral tissues, organs such as the heart and kidney showed no or minimal histopathology and infiltration. However, transcriptomic changes lasted for up to 31 days after infection in the olfactory epithelium and the olfactory bulb of infected hamsters, which caused changes in the behavior of hamsters, specifically a hypoexcitable state. Histological changes in the lung included enlarged airway spaces and residual inflammation characterized by monocytes and neutrophils in the alveolar spaces, which correlated with cluster of differentiation 177 (*Cd177)* and lymphocyte antigen 6 family member D (*Ly6D)* expression changes. No obvious signs of fibrosis or collagen deposition were evident (*74*).

A study performed by Carrau *et al.* (*75*) investigated the induction of an antiviral response during acute infection with SARS-CoV-2. For this, they used dexamethasone to delay the interferon response. This induced a delayed activation of an appropriate interferon response, which did not affect the overall viral load in the lungs of hamsters but delayed viral clearance. This delay in viral clearance allowed systemic seeding of SARS-CoV-2 to other sites and promoted the infection of distal organs such as the liver, kidney, spleen, and brain, thereby contributing to the development of Long Covid (*75*).

Zazhytska *et al.* (*76*) studied SARS-CoV-2–induced anosmia in more detail in the hamster model. The infection of hamsters with SARS-CoV-2 induced the recruitment of immune cells from the periphery to the olfactory epithelium and a rapid induction of an antiviral response (*76*). This hyperinflammatory response in the olfactory epithelium was accompanied by preferential infection and depletion of sustentacular cells, which were fully restored after 10 days of infection. In addition, down-regulation of olfactory receptors and key genes of olfactory receptor signaling pathways was detected, potentially explaining chronic anosmia phenotypes (*76*).

A study by Serafini *et al.* (*77*) demonstrated persistence of viral RNA at low frequencies in different brain compartments after SARS-CoV-2 infection in hamsters. After live virus clearance and by 31 days after infection, viral RNA persisted in the olfactory bulb, cortical areas, the brain stem, and the cerebellum. Transcriptomics revealed infiltration of replicating virus into the CNS and in sensory tissues as soon as 24 hours after intranasal inoculation of hamsters with SARS-CoV-2. Specifically, the cervical and thoracic spinal cord and dorsal root ganglia were affected, leading to altered neuronal signaling. At 31 days after infection, transcriptomics of thoracic dorsal root ganglia revealed the development of neuropathic signatures and SARS-CoV-2–specific mechanical hypersensitivity (*77*).

Rizvi *et al.* (*78*) demonstrated the development of cardiovascular complications in SARS-CoV-2–infected hamsters, which started to emerge 14 days after infection, and were characterized by ventricular wall thickening and interstitial coronary fibrosis. The development of cardiovascular complications was associated with elevated concentrations of serum cardiac troponin-l, cholesterol, low-density lipoprotein, and long-chain fatty acid triglycerides. *N*-acetylneuraminate was identified as a common marker for cardiovascular complication development in SARS-CoV-2–infected hamsters and humans (*78*).

Together, these studies demonstrate that the hamster can be deployed as a suitable model system to study certain aspects of Long Covid, including regeneration of the lungs, the development of cardiovascular complications, and long-term neurological perturbations. As of now, therapeutic regimens designed to reverse Long Covid phenotypes have not been intensely pursued in this species (Fig. 1), and a central question remains as to whether these early Long Covid disease signatures will progress over time or resolve.

## Ferret models

The ability to accurately model human respiratory disease is of fundamental importance to the evaluation of therapeutic interventions, including small-molecule inhibitors and vaccine platforms. The domestic ferret (*Mustela putorius furo*) presents as an intermediate-sized animal platform to study such diseases. Ferrets have been effectively used to study multiple respiratory diseases, including H1N1 influenza A virus (IAV), respiratory syncytial virus, and SARS-CoV, among many others (*79*). The similarities between the upper and lower respiratory systems of ferrets and humans provide some advantages for modeling respiratory infections and transmission pathways such as SARS-CoV-2, although symptomatic disease is limited. Chu *et al.* (*80*) identified that during SARS-CoV infection, viral RNA was detectable in the nasal and lung tissues for at least 7 days after infection and that infectious virus was cleared at this time. However, not all animals were productively infected in this study (*80*). Weingartl *et al.* (*81*) also demonstrated that SARS-CoV–infected ferrets did not display any overt clinical symptoms through 29 days of observation after infection, yet infection still elicited a low number of neutralizing antibodies. Viral RNA was present in both the blood and in pharyngeal swabs, with detection possible up to 22 days after infection, although the importance of these findings remains uncertain (*81*).

van de Ven *et al.* (*82*) demonstrated similar findings with SARS-CoV-2. In this study, intranasal and intratracheal inoculation did not elicit any overt symptoms characteristic of SARS-CoV-2 infection (*82*). Although replication-competent virus was cleared by 9 days after infection, viral RNA could still be detected by quantitative polymerase chain reaction at 21 days after infection. Pathological abnormalities were observed in nasal, tracheal, and bronchiole tissues of challenged ferrets. These findings, coupled with worsening pathological features between samples analyzed at 14 and 21 days after infection, led the authors to postulate that ferrets may serve as potential models of dyspnea observed in patients with Long Covid (*82*). Ryan *et al.* (*83*) also found concurring data that showed mild clinical signs of SARS-CoV-2 infection in ferrets, although outcomes were less severe than what was reported from studies with SARS-CoV. Viral RNA shedding from the upper respiratory tract was detectable through 14 days after challenge, and pathological lung abnormalities were also a tractable metric (*83*). Similarly, Au *et al.* (*84*) observed that there were no clinical signs of infection after SARS-CoV-2 challenge but that infectious virus could be recovered from oral swabs, nasal turbinates, and pharynx samples, although samples collected from ferrets at 14 days after

infection had no quantifiable viral RNA (Fig. 1). Longer-term studies are required to understand the relative strengths and weaknesses of this model.

Similar to the hamster, a strength of the ferret model is as a model of both direct and indirect transmission of SARS-CoV-2 between animals, although studies have come to mixed conclusions on the overt symptoms of disease noted in ferrets (*85*–*87*). Model variation could potentially be attributed to several sources, such as differences in experimental setup; viral isolate and inoculum used; or age, sex, or source of animals between studies. Transmission studies have identified the capacity of shed virus to be transmitted over varying distances to recipient animals, with spread noted in a system with a 1-m distance between the animals as confirmed by nasal and throat swabs. In summary, ferrets provide a model in which the infectivity of SARS-CoV-2 can be evaluated, in both the acute and chronic phases or through transmission studies where exposure can be achieved through direct or indirect contact (*88*). The accessibility and ability to perform nasal washes and throat swabs enable straightforward tracking of upper airway infections (Fig. 1). Further exploration of disease in ferrets may be of use in the assessment of virus reservoirs and complications resulting in Long Covid phenotypes after either direct or indirect infection.

### Nonhuman primate models

Nonhuman primates (NHPs)—including rhesus macaques, cynomolgus macaques, and African green monkeys—are important model species in research because of their physiological, immunological, and genetic similarities to humans. Despite limited numbers and high costs, specialized housing requirements, and highly trained animal care staff that can increase burden on researchers, these models are in high demand for pathogenesis model development. As such, these species have been of substantial importance in the face of SARS-CoV-2 because of their utility in preclinical studies for evaluation of vaccines and therapeutic interventions (*89*, *90*). The high amino acid identity between human and NHP entry receptors allows for the study of natural isolates without the need for virus adaptation or the development of transgenic models, although natural variants of ACE2 in some outbred NHP models have been shown to result in attenuated SARS-CoV-2 infection (*91*).

Assessment of infection in cynomolgus macaques infected with either wild-type ancestral or Omicron lineage SARS-CoV-2 found that, by 21 days after infection, all animals tested negative for SARS-CoV-2 RNA in both nasal and tracheal swabs and remained negative through 18 months after infection. Although the animals were negative for viral RNA in nasal and tracheal swabs, analysis of bronchoalveolar fluid macrophages identified that low amounts of viral RNA and spike protein persisted in more than half of the studied animals at 6 months after infection. These effects resulted in decreased interferon-γ production after stimulation with lipopolysaccharide and suggest a potential reservoir for viral persistence after the clearance of detectable viral RNA from the upper respiratory system (*92*). Additional studies are needed to confirm the prolonged persistence and potential replication of SARS-CoV-2 within bronchoalveolar fluid macrophages in infected macaques.

Although NHPs come with the previously mentioned constraints, they remain a valuable model to further study the impacts of Long Covid phenotypes. Further assessment of persistent viral reservoirs and the impact on resultant physiological symptoms makes them a compelling model to assess the efficacy of vaccines and small-molecule inhibitors as interventions for different Long Covid phenotypes. The increased life span of NHPs also enables long-term follow-up in both juvenile and adult animals, which is of high relevance for the human population. Further research into these areas should be pursued (Fig. 1).

## FUTURE DIRECTIONS

### The brain-lung axis and Long Covid

Neural networks sample and share critical homeostatic information between the lung and brain. The circadian molecular clock is disrupted under hypoxic conditions and in chronic pulmonary diseases (*93*). Disturbances in sleep, circadian rhythms, and daytime functioning occur in patients with Long Covid (*93*). In addition to the upper respiratory connection to the brain through OSN innervation of the CNS, the lungs are innervated by the vagus nerve, which has been shown to be involved in the pathogenesis of acute respiratory distress syndrome, pulmonary fibrosis, and numerous neurological conditions (Fig. 3) (*94*). Neuroendocrinology also plays an important role in stress responses and can contribute to long-term CNS and lung dysfunction. In the lung, pulmonary neuroendocrine cells monitor airway oxygen and respiratory status and rapidly signal this internal sensory information through afferent sensory neurons to the brain to regulate breathing. Pulmonary neuroendocrine cells express dozens of partially characterized neuropeptide and peptide hormone genes (*95*), including a variety of neuropeptides such as calcitonin gene–related peptide (CGRP), which can activate lung goblet and immune cells to promote asthma-like phenotypes. CGRP and neuropeptide Y can increase endothelium permeability, excess lung fluid, and hypoxemia, and antagonizing CGRP signaling was shown to improve barrier function and oxygenation (*96*). In humans, vagus nerve inflammation and autonomic dysfunction may also contribute to the dysautonomia observed in Long Covid (*97*). COVID-19 infection also reduces peripheral serotonin concentrations, which is thought to impair cognition through reduced vagal signaling (*57*).

Consequently, an emerging hypothesis is that hypoxic-ischemic injury, uncontrolled cytokine storm, chronic inflammation, neuropeptide release, and fibrosis in the lung may elicit persistent infection-induced changes in steroids, neuropeptides, and neurotransmitters. This in turn may promote cycles of persistent lung and CNS inflammatory disease and might also contribute to sex differences in the prevalence of Long Covid symptoms. The use of small-animal models to evaluate the connections and signaling between organ compartments may represent a rich area of future investigation into the mechanisms of Long Covid.

### The Collaborative Cross mouse genetic reference population

Current definitions of Long Covid disease phenotypes remain in a state of flux, hampering the identification of susceptibility loci that regulate chronic disease phenotypes. It is also likely that one or more Long Covid disease endotypes may occur in humans. At least 49 genes encode natural variation thought to promote acute SARS-CoV2 pathogenesis in humans, yet the role of these genes in Long Covid remains unexplored. A genome-wide association study for Long Covid identified the *FOXP4* locus as a potential driver of both acute and chronic disease (*98*). These data support the hypothesis that Long Covid is a complex disease that is regulated, in part, by host susceptibility loci. Consequently, rodent genetic reference

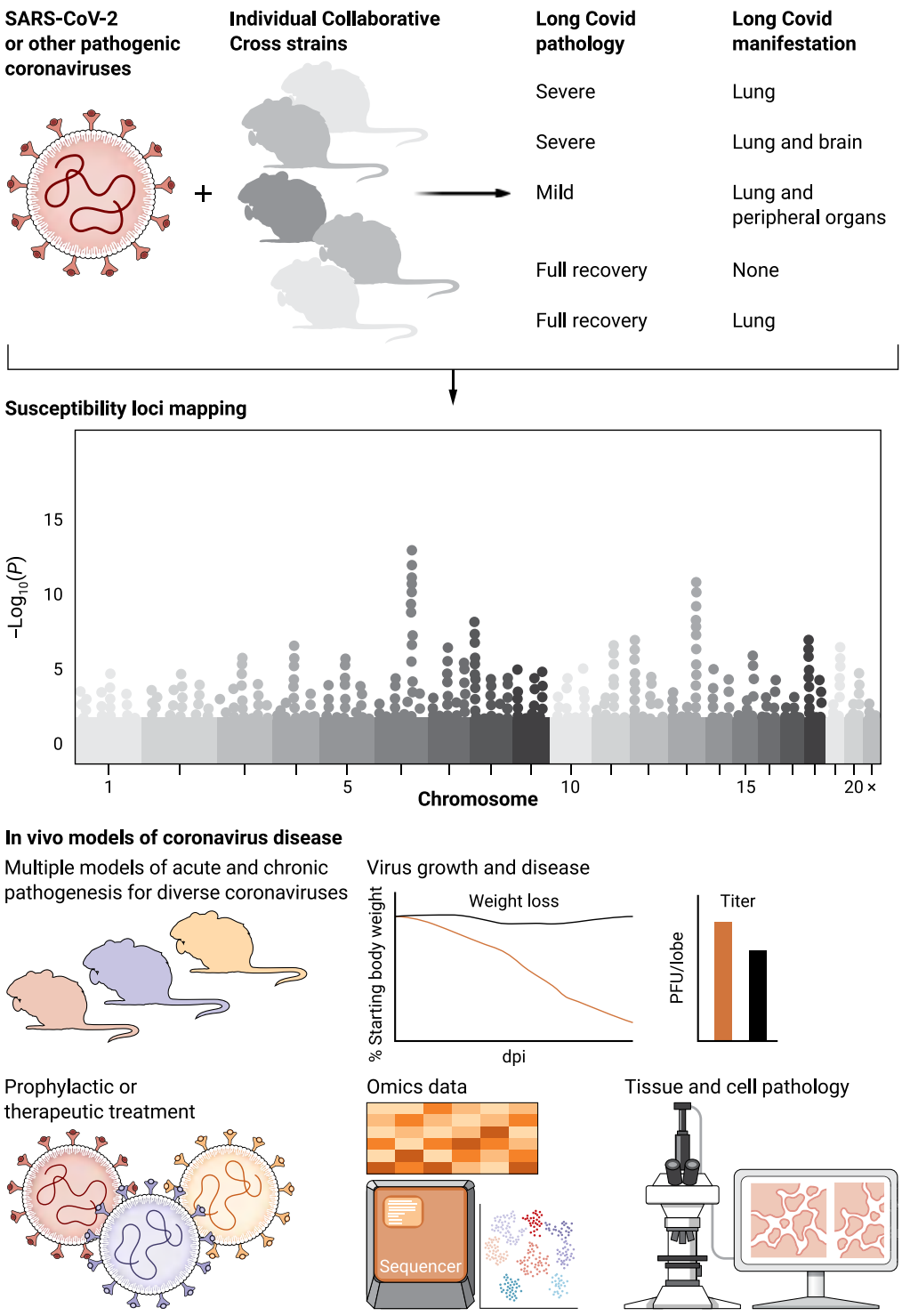



**Fig. 4. Leveraging the Collaborative Cross to study the effects of genetics on Long Covid development.** Depending on the development of Long Covid disease in individual Collaborative Cross strains after SARS-CoV-2 infection, those strains could be used as models for different aspects of Long Covid. Defined Collaborative Cross F2 crosses could be used to develop mapping crosses to identify host genetic networks that drive the development of certain aspects of Long Covid.

models may provide opportunities to identify highly vulnerable mouse strains with an array of related but different severe chronic disease endotypes. The Collaborative Cross is a mouse genetic reference population that was specifically designed for systems genetics and complex trait mapping. The Collaborative Cross is composed of eight founder strains: A/J, C57BL/6J, 129S1/SvImJ, NOD/ShiLtJ, NZO/HiLtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ. The founders were bred in an eight-way breeding funnel followed by brother-sister mating to produce about 80 inbred Collaborative Cross mouse strains, which are highly reproducible, tractable, genetically defined, and commercially available. Thus, each individual Collaborative Cross mouse strain contains randomly distributed genomic regions (e.g., scrambled susceptibility loci) from each of the eight parent strains and can be used to model how host genetic background and natural variation influences disease progression and outcome in outbred populations (*99*, *100*).

The Collaborative Cross resource has been used to successfully identify and validate susceptibility loci and genes that regulate sarbecovirus, IAV, Ebola virus, West Nile virus, and hepaciviruses replication and pathogenesis (*32*–*34*, *101*–*104*). After infection of Collaborative Cross–based large-scale screening populations with the same dose of mouse-adapted SARS-CoV-2, different Collaborative Cross strains experienced mild to severe life-threatening disease and mortality after infection, demonstrating that host genetic variation regulated disease severity. Using different Collaborative Cross strains, several common genomic regions or quantitative trait loci were identified that were associated with disease severity after SARS-CoV, SARS-CoV-2, and zoonotic CoV infection. About 60% of the defined murine quantitate trait loci encode genes that have been identified as prioritized candidates in human genome-wide association studies. These regions have been found to be associated with severe disease progression, the need for hospitalization and ventilation, and death in humans after SARS-CoV-2 infection (*32*–*34*, *105*). These data suggest that quantitate trait loci mapping studies may be fruitful because several screens in the Collaborative Cross have identified outcome models. Consequently, the Collaborative Cross may be an optimal platform to identify candidate mammalian genes that regulate Long Covid disease endotypes in mammals, providing critical resources for mechanistic studies and countermeasure development. The Collaborative Cross also represents a robust genetic resource for mapping candidate genes that drive Long Covid disease phenotypes specifically in the lung, brain, or other organs, including cognitive and behavioral changes after infection (*106*). In addition, the Collaborative Cross platform can be used to design specific mapping crosses to investigate, identify, and validate the causal molecular-genetic mechanisms that contribute to Long Covid outcomes across and between different organ systems (Fig. 4).

## SUMMARY AND THERAPEUTIC APPLICATIONS

Successful treatment of Long Covid disease phenotypes in humans is likely to be an expensive and sometimes difficult to quantify process that is complicated by highly variable risk factors (e.g., age, sex, comorbidities, genetics, etc.) and early-stage disease phenotypes that complicate measures of drug performance. Moreover, clinical trials may require years of follow-up in human populations to identify a clinical benefit, and the cost of such studies can be enormous. Animal models of Long Covid provide ample opportunities to identify early common disease correlates and molecular signatures associated with advanced disease development over time and also provide highly reproducible and tractable models to evaluate countermeasure performance. Such models can be manipulated to evaluate countermeasure performance before and during acute infection, as well as early or late in the development of mild to severe Long Covid. More broadly, these models will help to promote more aggressive research into the complex linkages between acute viral infection and long-term chronic disease manifestations.

The impact of repeat infection and vaccine breakthrough infections on Long Covid disease severity and progression has not yet been modeled and investigated using SARS-CoV-2 VOCs, other emerging sarbecovirus and merbecovirus infections, or other respiratory virus infections. Such studies are needed to not only test the hypothesis that repetitive injury and inflammatory insult enhance tissue remodeling and disease severity but also provide more relevant models for therapeutic intervention studies. Last, specific risk factors and combinations of risk factors can be isolated and evaluated to determine relative contributions to Long Covid, as well as the capacity of countermeasures to prevent and or reverse chronic outcomes in the background of preexisting, highly vulnerable states. Clearly, this rich research topic will reveal mechanisms regulating chronic organ diseases after viral infection while yielding insights into the pathways and genes that can be targeted by drugs that attenuate disease potential. At this point of Long Covid animal model development, none of the models discussed here has been sufficiently investigated. All models need to be studied in parallel because different model systems may capture different disease endotypes that require specifically targeted countermeasure designs.

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**Acknowledgments**
**Funding:** This work was supported by grants from the National Institute of Allergy and Infectious Diseases (NIH AI171292, AI157253, AI167966, and AI110700 to R.S.B.). **Competing interests:** R.S.B. is a member of advisory boards for VaxArt, Takeda, and Invivyd and has collaborative projects with Gilead, Johnson & Johnson, and Hillevax focused on unrelated projects. S.R.L. and R.S.B. are coinventors of methods and uses of mouse-adapted SARS-CoV-2 viruses (US Patent US11225508B1).

Submitted 14 April 2024
Accepted 16 October 2024
Published 13 November 2024
10.1126/scitranslmed.ado2104

LONG COVID

# Translating insights into therapies for Long Covid

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**Long Covid is defined by a wide range of symptoms that persist after the acute phase of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Commonly reported symptoms include fatigue, weakness, postexertional malaise, and cognitive dysfunction, with many other symptoms reported. Symptom range, duration, and severity are highly variable and partially overlap with symptoms of myalgic encephalomyelitis/chronic fatigue syndrome and other post-acute infectious syndromes, highlighting opportunities to define shared mechanisms of pathogenesis. Potential mechanisms of Long Covid are diverse, including persistence of viral reservoirs, dysregulated immune responses, direct viral damage of tissues targeted by SARS-CoV-2, inflammation driven by reactivation of latent viral infections, vascular endothelium activation or dysfunction, and subsequent thromboinflammation, autoimmunity, metabolic derangements, microglial activation, and microbiota dysbiosis. The heterogeneity of symptoms and baseline characteristics of people with Long Covid, as well as the varying states of immunity and therapies given at the time of acute infection, have made etiologies of Long Covid difficult to determine. Here, we examine progress on preclinical models for Long Covid and review progress being made in clinical trials, highlighting the need for large human studies and further development of models to better understand Long Covid. Such studies will inform clinical trials that will define treatments to benefit those living with this condition.**

## INTRODUCTION

Long Covid is defined by a wide range of systemic, neurologic, respiratory, cardiovascular, and other symptoms or conditions that continue or develop after the acute phase of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Although the duration of symptoms required to meet the definition of Long Covid is described variably, the World Health Organization (WHO) defines Long Covid as when symptoms not attributable to other causes are present more than 3 months from the onset of coronavirus disease 2019 (COVID-19) and last for at least 2 months (*1*). It has been estimated to occur in 6 to 30% of people who survive acute COVID-19 (*2*–*5*). The incidence of Long Covid is higher in people who had severe acute COVID-19 requiring hospitalization, and multiple other risk factors have been defined (Fig. 1) (*6*). However, because most people who experience COVID-19 do not require hospitalization, most people with Long Covid had mild to moderate illness in the acute phase of COVID-19 (*7*).

Long Covid symptoms can persist for weeks, months, or years, and the severity ranges from mild to debilitating. The impact of persistent disease in such a large proportion of the global population is enormous, with estimated economic impacts of more than 2 trillion US dollars in the United States alone (*8*). We now know that the increased risks of death and morbidity continue into the third year after infection in people who had severe acute COVID-19 (*9*), suggesting that the health impacts of having had COVID-19 may persist for years. Broadly, potential mechanisms of Long Covid include persistence of viral antigen or reservoirs, dysregulated immune responses (including autoimmunity and inflammation), direct viral damage of tissues targeted by SARS-CoV-2 during acute infection, inflammation driven by reactivation of latent viral infections, metabolic derangements, vascular endothelium activation or dysfunction, thrombosis, dysregulated complement, and microbiota dysbiosis (Fig. 2). Randomized controlled trials are urgently needed to identify effective new therapies. Here, we will review recent findings that have advanced understanding of mechanisms behind Long Covid, highlight opportunities and challenges for translating these findings into therapeutics, outline work done with animal models of Long Covid, and review progress on clinical trials for Long Covid.

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## CHALLENGES IN DEFINING THE MECHANISTIC UNDERPINNINGS OF LONG COVID

### Defining Long Covid with variability between individuals

Defining Long Covid in a consistent manner is challenging but necessary to advance mechanistic research and clinical trials. Groundbreaking early research by the Patient Led Research Collaborative for Long COVID found that some of the most common symptoms of Long Covid are fatigue, postexertional malaise, and cognitive dysfunction, and they noted a remarkably high average of 55.9 ± 25.5 (mean ± SD) symptoms in people with Long Covid across an average of 9.1 organ systems with a range of severities (*10*). Subsequent studies have confirmed these as common symptoms and found that some symptoms frequently cluster in individuals experiencing multiple symptoms (*11*–*14*). Preliminary results from more than 9000 adults in the US-based National Institutes of Health (NIH) RECOVER (Researching COVID to Enhance Recovery) cohort identified a classifier for Long Covid based on 12 symptoms using a data-driven scoring framework (*12*). Here, the most common symptoms reported by people with Long Covid at 6 or more months after infection were postexertional malaise (87%), fatigue (85%), brain fog (64%), dizziness (62%), gastrointestinal (GI) symptoms (59%), and palpitations (57%). Unsupervised learning techniques clustered people with Long Covid into four subgroups on the basis of self-reported symptoms. However, subgroups of Long Covid do not always align between studies, in part because of differences in data collection between studies. For example, the four Long Covid subgroups identified from self-reported symptoms in the adult RECOVER cohort have different phenotypic features than the six Long Covid subgroups identified

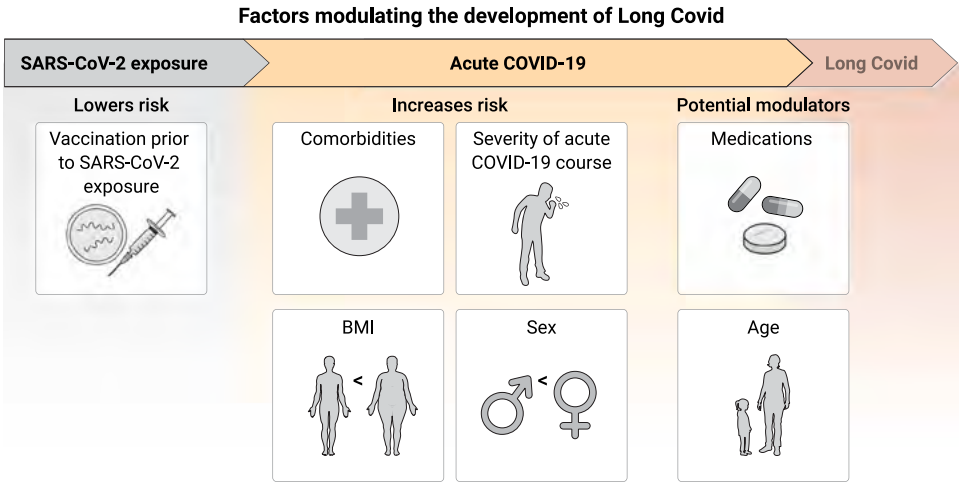



**Fig. 1. Several factors modulate Long Covid progression.** Increased acute disease severity, elevated BMI, female sex, and concomitant comorbidities increase the risk of Long Covid, whereas vaccination before infection decreases the risk. Treatments in the acute phase and age may also influence risk.

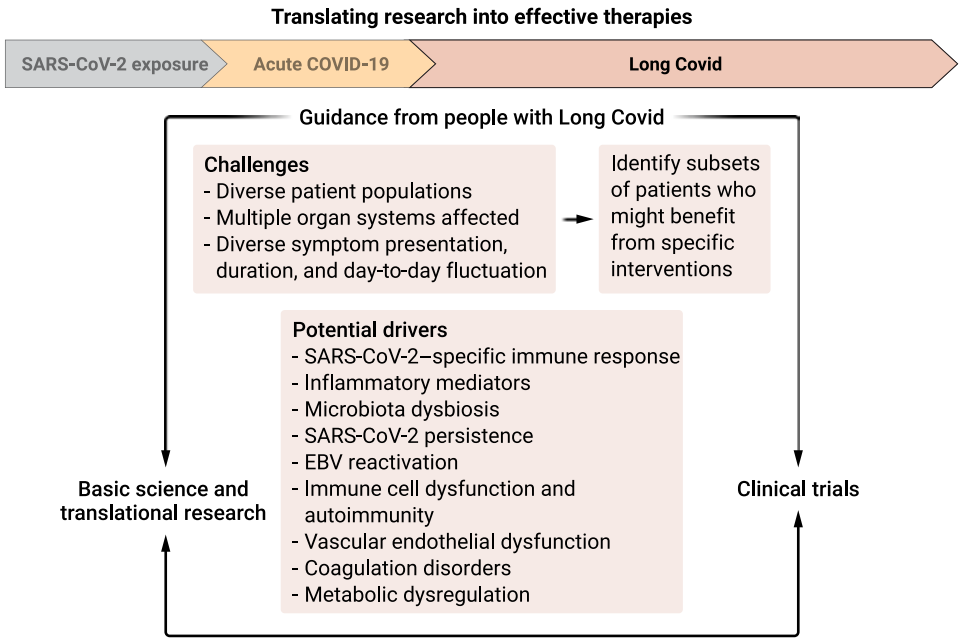



**Fig. 2. Translational research is required to identify effective therapies.** The diverse etiologies of Long Covid and patient input define research and clinical trial goals. The potential causes of Long Covid need to be characterized to identify the most promising treatments. Current clinical trials are detailed in the text.

from an electronic health record (EHR) study using the US National COVID Cohort Collaborative (N3C) EHR repository (*14*).

It is likely that there are both distinct and overlapping mechanisms for the different manifestations of Long Covid. Consideration of sex, age, body mass index (BMI), smoking status, vaccination status, severity of disease in the acute phase, viral variant, the presence or absence of a wide variety of underlying medical conditions, and other demographic variables is also required to understand factors that lead to Long Covid (Fig. 1). Therefore, studies with large numbers of participants are required to control for these factors. As previously reviewed, multiple epidemiologic studies have highlighted factors associated with Long Covid, for which mechanistic studies would ideally adjust (*15*–*19*). Factors like female sex, obesity, and severe acute disease are associated with higher risk of Long Covid, whereas COVID-19 vaccination before infection and young age are associated with lower risk of Long Covid (*12*, *20*–*31*).

Recent large studies have identified long-lasting impacts of SARS-CoV-2 infection on cognition and memory and suggest that the consequences of SARS-CoV-2 infection differ by baseline characteristics of infected individuals and by acute COVID-19 timing and severity (*32*–*36*). A study in Norway assessed memory by Everyday Memory Questionnaires (EMQs) for 57,319 individuals who tested positive for COVID-19 and 54,673 people with no positive tests for COVID-19 after baseline memory testing (*37*). Although EMQs were comparable at baseline, the tests revealed impaired memory in those who tested positive for COVID-19 as long as 36 months after infection versus those who did not test positive. Impaired memory was more common in younger individuals, those with a longer duration of being bedridden, those with a higher BMI, and women, consistent with previously described associations between elevated BMI or female sex and Long Covid (*15*–*19*). In a study of 112,964 people in England comparing scores from a single online cognitive assessment administered 4 to 32 months after infection, mild cognitive deficits were observed in those who had COVID-19 with symptoms that resolved within 1 or 3 months compared with those who never had COVID-19 (*33*). Increased cognitive deficits were associated with reporting persistent symptoms or infection earlier in the pandemic. This study is consistent with other large studies demonstrating decreased frequency of Long Covid after infection with SARS-CoV-2 variants of concern (VOCs) versus ancestral strains (*33*, *38*–*43*), although not all studies confirm this finding (*44*). That the most common symptoms of Long Covid occur after infections with many variants suggests effects of the virus shared by variants. The reduced rates of Long Covid seen with Omicron variants may be due to beneficial effects of previous immunity through vaccination or previous infection or due to effective treatments used later in the pandemic. Thus, a role for any distinct feature of SARS-CoV-2 variants in progression to Long Covid has not

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yet been defined. The relative benefit of being infected when later variants were circulating is likely mitigated at a population level by increased infectivity of the Omicron variant and relaxation of infection prevention measures, with the net result being a smaller percentage but larger numbers of infected people developing Long Covid.

### Defining Long Covid with variability within individuals

An additional challenge is that symptoms and sequelae vary in presence and severity over time within the same individual. Long Covid symptoms may occur or worsen with exercise, stress, or physical or mental activity (*10*). Whether fluctuations in symptoms are associated with the same magnitude or timing of changes in systemic immunity, inflammatory activation state, vascular endothelial activation or dysfunction, and other potential causes of Long Covid is unknown, making it difficult to identify associations. A tractable way to investigate this is with longitudinal studies of people with Long Covid, which are underway now. Another more controversial way to investigate this is to assess individuals before and after a stimulus to determine the magnitude and timing of changes in parameters of interest. A recent study identified skeletal muscle structural changes, severe exercise-induced myopathy, and tissue infiltration of amyloid-containing deposits in skeletal muscles of people with Long Covid that worsen after induction of postexertional malaise (*45*). However, the design of this type of study should be guided and advised by people living with Long Covid, and the potential benefit must be carefully weighed against the ability of people with Long Covid to participate and the potential for harm by inducing more severe symptoms.

## SUCCESSES IN DEFINING MECHANISTIC UNDERPINNINGS OF LONG COVID

An enormous debt of gratitude goes to the many people living with Long Covid who sounded the alarm about this new disease in the lay press, advocated for research funds, began the first large-scale studies themselves without the support of the traditional research enterprise, participated in studies to advance our understanding of the causes of Long Covid, and continue to guide the research field (*10*, *46*). Without the strong advocacy, hard work, and selfless collaboration of people living with Long Covid, the important discoveries made to date would not have been possible. We review here several candidate disease mechanisms for Long Covid.

### Persistent viral reservoirs

The evidence for persistent viral antigen or reservoirs and possible mechanistic links to Long Covid has been recently reviewed in depth elsewhere (*19*, *47*–*51*). Wastewater data and a large sequence repository analysis suggest that a small percentage (<1%) of people harbor actively replicating SARS-CoV-2 in the GI tract or nasal epithelium for 30 or more days (*52*, *53*). It remains unknown whether a similar percentage of people harbor actively replicating virus in other organs and whether persistent reservoirs are related to post-acute symptoms or sequelae. We will briefly review here the evidence of persisting viral antigen and reservoirs in humans in the circulation and the GI tract, given that these are commonly sampled in human studies.

SARS-CoV-2 can infect many tissues during acute infection (*54*). Several studies have found that the presence of viremia during acute infection is associated with infection severity (*55*–*59*). It is not yet known whether all infection events with SARS-CoV-2 have a viremic phase, with duration of viremia associated with severity, or whether only some infection events have a viremic phase. Swank and colleagues used an ultrasensitive immunoassay and found that 60% of 37 people with Long Covid had detectable plasma spike antigen 3 to 12 months after infection, although very few had detectable S1 (a subunit of the spike protein) or nucleocapsid (N) antigen (*60*). In another study, 64% of 22 people with Long Covid were found to have circulating S1 antigen as measured by enzyme-linked immunosorbent assay (ELISA), but more than 30% of 17 post–COVID-19 participants who never had Long Covid also had detectable S1 antigen, and whole spike and N antigen were not quantified (*61*). In a study of 171 people using the same ultrasensitive immunoassay, 25% of people were positive for any one circulating SARS-CoV-2 antigen in the post-acute phase, and the presence of post-acute SARS-CoV-2 antigen was associated with acute COVID-19 severity but not with Long Covid (*62*). Smaller studies reported that circulating spike antigen, viral RNA, or viral antigen in neuron- and astrocyte-derived extracellular vesicles or whole blood can be detected for months after infection and are associated with Long Covid (*63*–*65*). Two of these manuscripts report a higher prevalence of circulating antigen in people who had more severe acute disease, which may be consistent with severity of acute infection being associated with acute viremia and more widespread dissemination. It is important to note that detection of viral antigen may be compromised by shedding kinetics, assay sensitivity, and circulating antibodies. In sum, these data suggest that viral antigen can circulate in the bloodstream in the post-acute period, that it is associated with acute severity of illness, and that there may be an association of antigenemia with Long Covid, but that association is not yet established, and substantial numbers of people without Long Covid have circulating viral antigen as well.

Virus shedding from the GI tract in acute COVID-19 and persistence of viral antigen in the GI tract in the post-acute phase were noted early during the pandemic (*66*–*68*). Recent work used sequencing of wastewater samples to provide evidence of evolution of the virus in the GI tract (*52*). Peluso *et al.* describe imaging evidence suggestive of T cell activation in the gut wall, among other anatomic sites, in people in the post-acute period. They found viral RNA in the lamina propria of colorectal biopsies in people with Long Covid. However, biopsies of people without Long Covid who also had evidence of T cell activation in the gut wall in the post-acute period were not examined (*69*). Viral antigen in the lamina propria of the GI tract was also reported in a case report of an 11-year old child with persistent abdominal pain 3 months after SARS-CoV-2 infection (*70*).

A study of upper and lower endoscopic biopsies from 249 people a median of 17 (range, 7 to 30) months since last SARS-CoV-2 infection reported that SARS-CoV-2 N protein was detected in 37% of stomach biopsies and 17% of ileal and colon biopsies, but the presence of viral protein was not associated with time since infection or the presence of GI symptoms during acute COVID-19 (*71*). This study did not examine associations with Long Covid or persistent symptoms. A study of 46 people with inflammatory bowel disease undergoing intestinal biopsies, only 3 of whom required hospitalization for acute COVID-19, demonstrated that most (70%) had SARS-CoV-2 RNA in the gut mucosa on average 7 months after infection (*72*). Strikingly, all 21 participants with post-acute symptoms had detectable viral RNA in the gut, whereas 9 participants without post-acute symptoms had detectable viral RNA, and 14 other

participants without post-acute symptoms did not have detectable viral RNA. Viral N protein was found in gut epithelium, in $CD8^+$ T cells, and in the lamina propria in approximately 50% of participants, although the virus could not be cultured from tissue and viral antigen could not be isolated from stool. An interesting case series of five people undergoing GI/liver surgery with negative nasopharyngeal swabs 9 to 180 days after hospitalization for acute COVID-19 reported detection of SARS-CoV-2 N protein in all participants and viral RNA detection in two of five participants. Whether participants had Long Covid was not reported in this study. N protein was identified in colonic crypts and polyps; in immune cells, likely of monocytic lineage; and in sinusoidal Kupffer cells in the liver (*73*).

In sum, these studies demonstrate that viral protein and RNA can persist months or even years after infection in some people, but many questions remain: Which GI-resident cells and which regions of the GI tract harbor SARS-CoV-2 protein and RNA, do these cells support active viral replication, is fecal shedding of viral RNA sourced from actively replicating virus, does SARS-CoV-2 subvert viral clearance mechanisms, and do persistent viral antigens or RNA drive Long Covid symptomatology? Innovative strategies are needed to investigate how persistent viral antigen or reservoirs in human tissues may be mechanistically related to Long Covid; promising ones include novel radiological techniques (*69*), organoid model systems (*74*), sampling during other procedures, and autopsy studies of people with Long Covid.

## Dysregulated immune responses

Virus-specific immune responses have been investigated without finding consistent differences across studies. Whereas some studies have reported increased frequencies of spike protein– or N-specific antibodies in people with Long Covid, others have reported no change or decreases, making it challenging to define a proposed role for virus-specific antibodies in Long Covid (*75*–*81*). Similarly, studies have found completely different results in analyzing T cell responses to SARS-CoV-2, including no differences between those with and without Long Covid. Differences between people with Long Covid and those who recovered include a lower percentage of N-specific interferon (IFN)–$\gamma^+CD107a^+$ memory $CD8^+$ T cells in those with persistent symptoms (*77*), increased $CD4^+$ and $CD8^+$ T cell responses to peptide pools from spike (S), N, and membrane (M) proteins in patients with pulmonary Long Covid (*78*), and $CD8^+$ T cells that secrete less IFN-γ and tumor necrosis factor (TNF) in response to phorbol 12-myristate 13-acetate (PMA) (*82*). One study demonstrated that higher amounts of pain correlate with more interleukin-6 (IL-6) production by $CD8^+$ T cells in response to S peptide stimulation (*81*). Less is known about children with Long Covid (*83*), but a study by Paniskaki and colleagues demonstrated that children had higher frequencies of S protein–specific $CD4^+$ and $CD8^+$ T cells that secreted IFN-γ and TNF with low functional avidity (*84*). $CD8^+$ T cell production of IFN-γ was also correlated with C reactive protein (CRP). The lack of consistent differences in SARS-CoV-2–specific immunity between people with and without Long Covid among studies published to date could be explained by differences in participant populations, heterogeneity of immune responses, Long Covid immune subtypes, or small sample sizes.

A recent study of 40 people found evidence of activation of the complement system in people with Long Covid that spanned acute infection through 6 months of follow-up (*85*). People with Long Covid had evidence of dysregulation of the terminal portion of the complement cascade and activation of the alternative and classical complement pathways, the latter associated with elevated antibody titers against multiple herpesviruses that might be stimulating this activation. Complement activation normalized in those whose symptoms of Long Covid resolved at 6 months.

Given that only a small fraction of B and T cells are specific for SARS-CoV-2, alterations in the overall frequencies of immune cells would not be due to alterations in virus-specific immune cells (*86*–*88*). However, multiple groups have reported global alterations in immune cell populations in Long Covid, including activated myeloid subsets. Phetsouphanh *et al.* reported that $CD38^+$ human leukocyte antigen (HLA)–$DR^+$–activated myeloid cells were elevated in patients with Long Covid (*89*). A large-scale proteomic study of 657 posthospitalization participants found elevated concentrations of proteins associated with myeloid inflammation to be enriched in those with Long Covid, supporting that myeloid activation and inflammation are associated with Long Covid (*90*). Moreover, higher frequencies of T cell subsets with increased expression of programmed death-1 (PD-1)– and T cell immunoglobulin and mucin-domain containing-3 (TIM3)–expressing cells have been reported by multiple groups (*78*, *80*, *91*). Expression of both increases with T cell receptor (TCR) stimulation, and prolonged up-regulation is associated with T cell exhaustion. In the absence of functional analysis, it is not certain whether higher frequencies of exhausted T cells are found in people with Long Covid. In sum, although multiple studies show evidence of prolonged systemic immune dysregulation after COVID-19, the relationship between Long Covid symptoms and immunologic alterations is not clear.

## Inflammation and plasma inflammatory biomarkers of Long Covid

Inflammation is a driver of multiple disease processes that occur commonly after COVID-19. As one example, data from large health care systems demonstrate an increased risk of cardiovascular events in the 12 months after SARS-CoV-2 infection (*92*, *93*). Whether this is an effect of activation of inflammatory pathways linked to myocardial infarction or direct damage to the heart is not known.

Multiple cytokines and chemokines have been shown to be elevated in peripheral blood in patients with Long Covid (*94*). Multiple groups have found elevated IL-6 in blood from people with Long Covid (*78*, *89*, *95*). Elevated CRP has also been associated with Long Covid in some studies (*78*, *94*, *96*–*99*), but not all (*100*). Phetsouphanh and colleagues demonstrated that the combination of IFN-β2, IFN-γ, IFN-λ2/3, IL-6, and pentraxin 3 identified those with Long Covid at 8 months after COVID-19 with 79 to 82% accuracy (*89*). Additionally, Wang and colleagues' network analysis of cytokine, proteome, and metabolome differences between acute and convalescent patients with COVID-19 revealed sustained inflammatory responses, platelet degranulation, and cellular activation and multiple distinct metabolites (*101*). Their prognostic model composed of 20 molecules regulating metabolism and T cell exhaustion predicted adverse outcomes after acute COVID-19 with 83% accuracy. However, the predictors identified in these two studies were not concordant. The chemokine CCL11 (also known as eotaxin) has been shown to be elevated in blood from people with cognitive impairment, but not with other symptoms of Long Covid, and decreased with resolution of cognitive symptoms (*102*). CCL11 concentrations were also increased in the cerebrospinal fluid (CSF) in a mouse model of Long Covid, and

administration of CCL11 to mice was sufficient to induce microglia and macrophage reactivity in hippocampal white matter; this is interesting because hippocampal changes have been observed in magnetic resonance imaging (MRI) of people after COVID-19 (*102*, *103*). These data suggest that CCL11 might be an important marker of neurocognitive Long Covid.

### Autoimmunity

An increased risk of multiple autoimmune diseases, including type I diabetes mellitus (T1DM), systemic lupus erythematosus, rheumatoid arthritis, various forms of vasculitis, and inflammatory bowel disease, has been noted after SARS-CoV-2 infection. A recent meta-analysis demonstrated that the risk of new-onset T1DM after SARS-CoV-2 infection in children and adolescents was 42% higher compared with that in non–COVID-19 control groups (*104*). A study of 641,704 individuals who had COVID-19 revealed a 43% increased risk of autoimmune conditions during follow-up 3 to 15 months after documented SARS-CoV-2 infection (*105*). Likewise, another study evaluating the medical records of more than 22 million Asian patients noted an increased risk of autoimmunity of 25% in South Korea and 79% in Japan in individuals who had COVID-19 compared with uninfected controls (*106*). These rates were also higher than in people after influenza. Although vaccination reduced the risk of postinfection autoimmunity, overall rates were still increased after COVID-19 compared with controls.

Autoantibodies of diverse specificities have been identified in both acute COVID-19 and Long Covid and have been detailed in an excellent 2023 review (*107*). Most studies have not found a correlation between specific autoantibodies and specific Long Covid symptoms, nor have they found that resolution of Long Covid is associated with changes in autoantibody titers or specificity (*80*, *108*–*111*). However, Su and colleagues demonstrated that antibodies against IFN-α2 were correlated with Long Covid pulmonary disease (*75*). Two recent preprints reported that, in some cases, injection of mice with antibodies from people with Long Covid can induce heightened pain sensitivity (*112*, *113*). However, neither study identified specific autoantibodies that mediated this heightened pain sensitivity. One study revealed an association of antinuclear antibodies with persistent Long Covid symptoms 12 months after acute infection (*114*), and another showed that autoantibodies against multiple G protein–coupled receptors (GPCRs) could be detected in all 31 people with Long Covid enrolled in the study (*115*). Autoantibodies against GPCRs have been linked to postural orthostatic tachycardic syndrome (POTS) and dysautonomia (*116*–*118*), both of which are sequelae of Long Covid. However, the GPCR study did not link the antibodies to specific symptoms, and the assessment was done at a single time point, so whether onset or resolution of symptoms in Long Covid is associated with the presence of these anti-GPCR antibodies is unclear.

### Direct viral damage of tissues targeted by SARS-CoV-2 in acute COVID-19

Long Covid is a systemic disease involving multiple organs, as suggested by symptomatology and demonstrated in imaging studies. Focusing on two tissues in the respiratory tract that contain cells directly infected by the virus, the nose and lung, illustrates mechanisms behind prolonged effects of the virus. As reviewed recently, the breathlessness or dyspnea commonly reported in the post-acute phase may in some cases be due to pulmonary fibrosis and thrombotic disease (both pulmonary emboli and microvascular thrombosis), both of which are now recognized to be triggered during acute COVID-19 (*119*). In one study, $^{129}$Xe MRI and computed tomography (CT) pulmonary vascular measurements suggested that reduced pulmonary gas exchange, in association with dyspnea and reduced exercise tolerance, may be a pathogenic mechanism in Long Covid (*120*). Another study using dual-energy CT demonstrated perfusion defects in the lungs of approximately 60% of those with respiratory symptoms of Long Covid at 3 months after infection (*121*). Filling defects were observed in the pulmonary arteries, providing evidence of macrothrombosis (clotting) in 5% of patients (*121*).

Although SARS-CoV-2 targets the nasal olfactory epithelium, which includes olfactory sensory neurons, most evidence suggests that olfactory neuron infection is extremely rare, as is infection of the brain, implying that neuronal infection is not the most likely cause of anosmia in individuals with COVID-19 (*122*). Finlay and colleagues demonstrated that T cell–mediated inflammation in the olfactory epithelium, as well as inflammatory responses in sustentacular cells (supporting cells of the olfactory sensory neurons), was associated with a reduction in the number of olfactory sensory neurons and persistent anosmia (*123*). Other work has demonstrated that SARS-CoV-2 can mediate destruction of sustentacular cells (*124*). Among other reasons, the duration of anosmia may thus depend in part on how long it takes to regenerate sustentacular cells and restore functional olfactory neurons. As in the link between sustentacular cell destruction and anosmia, elucidating the effects of acute viral infection will also likely illuminate what enables symptom persistence.

### Inflammation driven by reactivation of latent viral infections

The infectious mononucleosis syndrome that follows Epstein-Barr virus (EBV) and, less commonly, cytomegalovirus (CMV) infection is, like Long Covid, a post-acute infection syndrome characterized by fatigue and myalgia. EBV and CMV establish latency after initial infection and can reactivate in the setting of acute infection, severe illness, or with immunosuppression (*125*, *126*). Multiple studies have identified evidence of EBV reactivation in COVID-19. Detectable EBV viremia in acute COVID-19 was predictive of persistent symptoms, and increased EBV early antigen-diffuse and viral capsid antigen seroreactivity has been found to be associated with Long Covid (*75*, *127*–*129*). However, these are correlations, and it is not known whether EBV reactivation is causing some or all of the symptoms of Long Covid, particularly given that EBV reactivation occurs in multiple forms of immune dysregulation. Conversely, reactivation of CMV and other herpesviruses has not been clearly associated with Long Covid (*75*, *128*, *129*). Investigation of the pathways that permit EBV reactivation could highlight factors important in latency reversal. Development of a wider array of effective, well-tolerated therapies directed at EBV could have additional benefits in other EBV-mediated disease settings.

### Coagulopathy and vascular endothelium activation or dysfunction

There is substantial evidence that people remain at increased risk of thromboembolic events for an extended period of time after COVID-19. A systematic review and meta-analysis over a mean follow-up period of 8.5 months after COVID-19 demonstrated that pulmonary embolisms were 3.16 times more likely after COVID-19, and deep venous thromboses were 2.55 times more likely (*130*). Dysregulation of serum complement and hemostatic factors may

contribute to a subset of Long Covid manifestations. As noted earlier, Cervia-Hasler and colleagues observed increased complement activation that persisted in people with Long Covid through 6 months after infection (*85*). Conversely, people with Long Covid experiencing symptomatic improvement before their 6-month follow-up had substantial reductions in complement activity. This study also demonstrated elevated tissue injury markers in blood and a thromboinflammatory signature characterized by markers of endothelial activation (including von Willebrand factor), platelet activation, and monocyte-platelet aggregates. Moreover, Liew and colleagues profiled proteins in 627 post-hospitalization participants and found that markers of complement activation were associated with development of Long Covid (*90*). Mroueh and colleagues also reported increased von Willebrand factor and platelet activation in porcine coronary artery endothelium exposed to plasma from SARS-CoV-2–infected individuals versus healthy controls (*131*).

Additional groups converged on alterations in complement and hemostasis in Long Covid. Jang and colleagues showed that those recovering from COVID-19 demonstrate a reduction in complement and coagulation cascades and enrichment for fibrin clot formation and fibrinolysis (*132*). In 1837 people hospitalized for COVID-19, Taquet and colleagues reported that elevated ratios of fibrinogen and D-dimer to CRP during acute hospitalization were associated with worse cognitive outcomes at 6 and 12 months (*133*). Multiple studies have demonstrated amyloid fibrin(ogen) particles (termed microclots) in the plasma of patients with Long Covid, but the authors also found that other conditions are also associated with these findings, and it is not clear that the microclots are responsible for symptoms (*134*, *135*). Finally, a plasma proteomic profiling study showed that vascular inflammation and a vascular proliferative state were both associated with Long Covid (*136*).

### Dysregulated metabolites and vagal signaling

Another potential hypothesis is that Long Covid is driven by serotonin, a monoamine neurotransmitter that can be sequestered within dense granules of platelets or converted to 5-hydroxyindoleacetic acid (5-HIAA) by plasma monoamine oxidases (*137*). Wong and colleagues identified that serotonin concentrations were depleted in acute COVID-19 and that low serotonin was associated with Long Covid in two separate cohorts (*137*). Mouse models suggest that this depletion is driven by a type I IFN response to viral RNA, by diminished uptake of its precursor tryptophan, and by hypercoagulability. However, Wong and colleagues found no differences in serotonin between people with and without Long Covid in a third cohort that had on average fewer symptoms of Long Covid than the first two cohorts they examined (*137*). Serotonin concentrations negatively correlated with numbers of symptoms, and serotonin concentrations during acute infection could not predict future Long Covid status in a fourth cohort (*137*). Their work suggests that decreased serotonin concentrations may impair cognition through reduced vagal signaling, but more studies are needed to confirm a role for serotonin in Long Covid.

### Cortisol and the hypothalamic-pituitary-adrenal axis

Cortisol is the major glucocorticoid hormone in humans and is synthesized in adrenal cortex cells of the adrenal glands. Several pathogens are known to cause adrenal insufficiency through a variety of mechanisms (*138*, *139*). Human adrenal cortical cells express the SARS-CoV-2 receptor angiotensin-converting enzyme 2 (ACE2) and co-receptor transmembrane protease, serine 2 (TMPRSS2) (*140*, *141*) and thus are susceptible to infection. Six autopsy case series of people who died with acute COVID-19 reported evidence of adrenal damage, SARS-CoV-2 infection of the adrenal glands, or both (*140*–*145*). Given that the symptoms of adrenal insufficiency overlap with those reported by people with Long Covid (*146*, *147*), and because hypocortisolism has been observed in chronic fatigue syndrome (*148*), several groups have examined cortisol concentrations and adrenal function in the post-acute period.

Two studies from independent groups demonstrated that low cortisol concentrations were associated with Long Covid. In the first, a screen of 454 plasma proteins identified lower amounts of cortisol and cortisone as two of the top three differential proteins in people with persistent symptoms versus those with complete recovery 2 to 3 months after COVID-19 symptom onset (*75*). In the second, investigators found that lower serum cortisol levels were the most significant individual predictor of Long Covid in unbiased machine learning models trained to identify distinguishing features of Long Covid. Of note, these models incorporated proteomic profiling data, immune cell subset analyses, cytokine analyses, autoantibody screens, and antibody reactivity to SARS-CoV-2 and other human viruses such as EBV (*80*). However, cortisol is secreted in a diurnal fashion with peak concentrations at awakening. These studies assessed cortisol at random times throughout the day, meaning that these studies could not provide a clear picture of the peak, trough, or diurnal patterns of cortisol secretion in people with and without Long Covid.

Other studies have not clearly corroborated the negative association of cortisol and Long Covid symptoms. Although one small study observed lower cortisol concentrations during an insulin tolerance test among people with Long Covid (*149*), another study reported higher amounts of cortisol in people with Long Covid–associated fatigue and depressive symptoms (*150*). Two other studies reported negative or mixed results (*151*, *152*). Two additional reports tested people with and without Long Covid using the standard clinical test of adrenal function, the adrenocorticotropin (ACTH) stimulation test, which tests the capacity of the adrenal gland to secrete cortisol after stimulation by exogenous ACTH. In one group of 84 people with Long Covid, none had abnormal ACTH stimulation test results (*153*). Similarly, a smaller study found no differences in morning cortisol or ACTH stimulation testing between people with and without Long Covid (*154*). In sum, ACTH stimulation testing suggests that frank adrenal insufficiency is not a common feature of Long Covid. Studies specifically designed to assess peak and trough cortisol concentrations are needed to properly assess the link between cortisol and Long Covid.

### The microbiome

Studies have shown that gut microbiota alterations are associated with Long Covid, which highlighted probiotic administration as a potential intervention (*155*–*159*). Immune responses to, and possible gut translocation of, fungal microbiota may also play a role in Long Covid pathogenesis (*160*, *161*). However, more studies are needed to clearly understand if and how the microbiome may play a role.

## ANIMAL MODELS OF LONG COVID

Several animal models are being developed to shed light on the pathogenesis of Long Covid. Development of mouse models of SARS-CoV-2 pathogenesis has not been straightforward, because

ancestral SARS-CoV-2 does not bind mouse ACE2, and thus common laboratory strains of mice cannot be infected by the ancestral strain (*162*, *163*). Instead, human ACE2 (hACE2)–transgenic mice, hACE2-transduced mice, mouse-adapted SARS-CoV-2 strains, and mouse hepatitis virus (MHV; another betacoronavirus) have been used to model SARS-CoV-2 pathogenesis (*164*). One common strain in the SARS-CoV-2 model work is the *K18*-hACE2–transgenic mouse (*165*), in which hACE2 is under the expression of an epithelial cell promoter (*Krt18*), allowing expression of hACE2 in airway and other epithelial cells. In addition, the Alpha variant and other N501Y-carrying variants of SARS-CoV-2 can infect wild-type mice (*166*), allowing simpler mouse model systems. Another common model system is the golden or Syrian hamster, which is naturally susceptible to SARS-CoV-2 (*167*). Finally, nonhuman primates have been used to model several aspects of SARS-CoV-2 infection. A challenge with animal models is in identifying objective measures that correlate with patient-reported symptoms. Animal models must assess measures other than symptoms to identify post–COVID-19 pathology, some of which may not correspond well to symptoms people report. We focus here on studies that examine animals 30 or more days after infection.

### Pulmonary Long Covid

Pulmonary Long Covid primarily manifests with dyspnea, or shortness of breath, with a smaller proportion of people also complaining of cough. Although a substantial proportion of people with pulmonary Long Covid have abnormalities on pulmonary function tests or imaging in the post-acute period, there are many people with pulmonary Long Covid who have normal results on objective tests and imaging (*168*). It is difficult to model dyspnea in the absence of objective findings, but progress has been made using animal models examining the pathogenesis of fibrotic lung lesions and delayed repair. In one study using a mouse-adapted SARS-CoV-2 strain (MA10) in wild-type mice, pulmonary alveolar, but not bronchial, lesions persisted beyond viral clearance and were associated with temporary secretion of proinflammatory and profibrotic cytokines (*169*). Even after cytokine concentrations normalized, spatial transcriptomics demonstrated prolonged upregulation of cytokine signaling, including transforming growth factor–β (TGF-β), in fibrotic regions of the lung. This post-acute pulmonary fibrosis was attenuated with early administration of an antiviral or an antifibrotic agent. Examining *K18*-hACE2–transgenic mouse lung tissue up to 45 days postinfection, another study reported that pulmonary progenitor cell proliferation in the regions of injured lung was decreased after SARS-CoV-2 versus influenza infection (*170*). Here, viral RNA was observed in lung tissue and S protein in pulmonary macrophages at 21 days, but viral subgenomic RNA (indicative of recent replication) was absent from lung tissue at this and later time points.

Post-acute lung pathology may be prevented or reversed. Another study using a model of Omicron infection of ACE2-transgenic mice provided convincing evidence that vaccination 30 days before infection may prevent perturbations in immune cell subsets present in lung tissue (*171*), consistent with clinical data that vaccination before primary infection prevents Long Covid. It is not yet clear whether a longer interval between vaccination and infection would provide the same protection. In another study that reported the development of a humanized mouse model of post–COVID-19 lung fibrosis, combined blockade of inflammation and fibrosis with anti-CD47 and anti–IL-6 was shown to reverse fibrosis (*172*).

### Neurological Long Covid

Many groups are developing animal models that recapitulate neurological Long Covid in the absence of severe acute infection. In one study of eight aged nonhuman primates infected with ancestral SARS-CoV-2, neuroinflammation, microhemorrhages, microthrombi, and evidence of hypoxic-ischemic injury were observed in central nervous system (CNS) tissues 28 days after infection (*173*). Virus was rarely detected 28 days after infection except in brain endothelial cells and was not found in CSF. Similarly, in a study of rhesus macaques infected with Delta and followed up to 50 days after infection, weekly positron emission tomography (PET) scans indicated active and increasing neuroinflammatory responses at time points at which most virus had likely been cleared from the CNS and its vasculature (*174*).

Frere and colleagues infected golden hamsters with SARS-CoV-2 and investigated tissue histology, tissue transcriptional profiles, and behavior up to 31 days after infection (*175*). Viral subgenomic RNA was detected early in the olfactory bulb, cerebellum, and striatum but was undetectable by 14 days after infection. Despite viral clearance, microglial activation, infiltrating macrophages, persistent type I IFN signatures, and other post-acute regional transcriptional changes were observed in the thalamus and the olfactory bulb. In another study, evidence of post-acute microglia and macrophage reactivity in the CNS was observed in hACE2-transduced (trachea and lungs) mice infected with SARS-CoV-2; this was also seen in uninfected mice administered CCL11 (eotaxin-1) (*102*). In one *K18*-hACE2 mouse study, viral protein and RNA did not persist in the post-acute period, but changes suggestive of microglial activation and reduced microglial processes were observed (*176*). Another *K18*-hACE2 mouse study confirmed that infectious virus and viral RNA were not detected in the CNS 1 and 4 months after infection despite autonomic and neurologic abnormalities, and activation of immune pathways was observed in transcriptomic analyses of CNS tissue (*177*).

Cheong and colleagues infected wild-type mice with MHV-1 as a model of betacoronavirus infection. They found that, at 30 days after infection, MHV-1 infection induced immunologic changes and demyelination in the brain. These changes could be at least partially blocked by early administration of IL-6 receptor (IL-6R) antibody, suggesting that IL-6 signaling during acute infection may be associated with post-acute immune dysregulation and pathology (*178*).

### Post-acute effects on the renal, cardiovascular, and somatosensory systems

Fewer groups have investigated the post-acute effects of COVID-19 on other organ systems. One group examined MHV-1–infected female mouse kidneys 12 months after infection and found inflammation, infiltration of macrophages and lymphocytes, and other changes suggestive of renal fibrosis, which were decreased by synthetic peptide–mediated inhibition of spike protein binding to ACE2 during acute infection (*179*). Another group used golden hamsters and guinea pigs to examine cardiac conduction system sequelae during acute and post-acute COVID-19 through 30 days after infection, finding that arrhythmias occurred during acute infection, then resolved, and subsequently could recur in the post-acute period (*180*). No viral RNA was detected in myocardial tissue.

Instead, cardiac innate immune responses were likely the proximate cause of dysrhythmias. Inhibition of Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling or the use of a mitochondrial-targeted antioxidant during acute infection ameliorated specific cardiac dysrhythmias. Many people with Long Covid complain of pain or abnormal skin sensations. Serafini and colleagues demonstrated, using a golden hamster model of SARS-CoV-2 infection, that post–COVID-19 mechanical hypersensitivity was associated with a neuropathic transcriptome from thoracic dorsal root ganglia (*181*). Moreover, they demonstrated that interleukin enhancer binding factor 3 (ILF3) inhibition suppressed IFN-induced hypersensitivity, which may be a promising target for Long Covid–associated pain.

## EARLY VIRUS-HOST KINETICS AND LONG COVID

### Acute phase viremia, delayed viral clearance, and viral load associate with Long Covid

Studies demonstrating the association between COVID-19 vaccination before infection and lower rates of Long Covid were the first hints that SARS-CoV-2 virus-host kinetics during the acute phase may be linked in a mechanistic way to the persistence of symptoms and development of sequelae in the post-acute phase. The first published report to demonstrate this linkage directly was by Su and colleagues, who found an association between SARS-CoV-2 RNAemia at the earliest blood sampling (average 13 days after infection, 209 participants) and self-reported memory problems 2 to 3 months later, after adjusting for age, sex, and respiratory support during acute COVID-19 (*75*). However, people who require hospitalization for acute COVID-19 are more likely to have Long Covid than those with mild to moderate infection, and a higher percentage of the people with RNAemia were ever hospitalized or in the intensive care unit (ICU) than in the group without RNAemia. So, it remained unclear whether the association of RNAemia with neurological Long Covid was really due to an association of acute severity with Long Covid. However, this finding was confirmed soon after in another posthospitalization cohort (*182*).

It remained to be seen whether the link between acute viral-host kinetics and Long Covid held true in people who were never hospitalized during acute COVID-19. Using longitudinal reverse transcription polymerase chain reaction (RT-PCR) of nasal swabs and saliva in individuals who did not require hospitalization during acute infection, our group demonstrated that delayed clearance of SARS-CoV-2 RNA from the upper respiratory tract during acute infection was associated with brain fog and muscle pain reported 90 or more days after symptom onset, providing more evidence that delayed or dysfunctional mechanisms of viral clearance are associated with Long Covid, specifically neurological Long Covid (*183*). This finding was affirmed later in a study of more than 1200 health care workers, in which self-reported SARS-CoV-2 positivity duration was associated with development of Long Covid, and in two cohorts of early-sampled nonhospitalized people (*184*–*186*). An additional longitudinal study of 590 unvaccinated people found that higher amounts of SARS-CoV-2 RNA in nasal swabs collected during hospitalization for acute COVID-19 were associated with the presence of two subtypes of Long Covid 3 or more months later (*187*). From these and other data (*53*), it seems clear that more total virus and more persistent virus during acute SARS-CoV-2 infection are associated with subsequently developing Long Covid, regardless of whether the virus persists past the acute infection.

### Acute antibody responses and their association with Long Covid

On the host side of the virus-host dynamic, several studies have shown a link between acute antibody kinetics and Long Covid. In one study of 116 people hospitalized in early 2020 with acute COVID-19, lower peak titers of blood anti-S immunoglobulin G (IgG) correlated with higher symptom burden at 2 and 6 months after infection (*76*). Similarly, a larger study of 304 mostly unvaccinated people with varied severity of acute COVID-19 found an association of lower titers of blood anti-N IgG titers at diagnosis with slower resolution of symptoms (*188*). Our study focusing on acute mucosal immune responses in 257 mostly nonhospitalized people with acute COVID-19 demonstrated that lower peak oral fluid anti-S and anti–receptor binding domain (S RBD) IgA and secretory IgA (sIgA) titers were associated with symptoms persisting beyond 4 weeks after infection (*189*). Here, higher peak oral fluid anti-S IgG titers were associated with longer time to symptom resolution, conflicting with the association of lower anti-S IgG with Long Covid described above. Together, these data support a model in which rapid and robust antibody responses contribute to timely clearance of the virus and limit viral dissemination during acute infection, whereas infections in which antibody responses are inhibited or viral clearance is delayed are more likely to be associated with development of Long Covid.

## PROGRESS TOWARD LONG COVID THERAPEUTICS

### Vaccination as a Long Covid therapeutic

Unfortunately, progress in identifying pharmacologic interventions for Long Covid has not kept pace with patient demand. Not long after the first COVID-19 vaccines were distributed to the general population, reports of the alleviation of some Long Covid symptoms after SARS-CoV-2 vaccination surfaced (*190*). An observational study of more than 28,000 people with previous SARS-CoV-2 infection by the UK Office for National Statistics reported in 2022 that the odds of experiencing Long Covid decreased 13% with a first vaccine and decreased a further 9% with a second vaccine in the post-acute phase (*191*). Survey-based studies observed that a higher proportion of people with Long Covid reported an improvement in symptoms after vaccination (14 to 86% depending on the study), although a significant proportion reported no change in symptoms (15 to 83%, depending on the study) (*192*–*195*). There have been no published randomized clinical trials of vaccination as a therapeutic for Long Covid, but in a trial emulation study of an online Long Covid cohort in France, Tran and colleagues followed 455 people with Long Covid before May 2021 for 120 days after the first SARS-CoV-2 vaccination and compared them with 455 propensity score–matched Long Covid controls who did not receive a vaccine (*196*). Having received a vaccine was associated with twice the rate of Long Covid resolution at the end of 120 days of follow-up, and those who received a vaccine reported less impact of Long Covid on their lives. In a small observational study examining biological correlates of improvement, prevaccination elevations in soluble IL-6R (sIL-6R) were associated with Long Covid symptom improvement, and elevations in IFN-β and ciliary neurotrophic factor (CNTF) were associated with no improvement after vaccination (*197*). Overall, these data indicate that some people with Long Covid may report at least temporary improvement after SARS-CoV-2 vaccination, but many people will report either no change or a deterioration in

symptoms, and therefore, randomized clinical trials of vaccines for Long Covid are needed.

### Clinical trials for Long Covid

Thus far, there have been few published randomized controlled clinical trials of pharmacological interventions for Long Covid. In the largest and most successful clinical trial to date, a randomized, double-blind, placebo-controlled trial of SIM01 (three lyophilized strains of *Bifidobacteria* and three prebiotic compounds) taken twice daily in 463 people starting at approximately 4 months after acute COVID-19, fewer people in the SIM01 group reported fatigue, cognitive problems, pain, insomnia, and GI upset at the end of 6 months of treatment (*198*). Increased bacterial richness and diversity and fewer bacterial antimicrobial resistance genes were found in microbiome analyses of the SIM01 group compared with placebo. Similar percentages in each group reported adverse events, which were all thought unlikely to be related to SIM01. Ideally, SIM01 should be tested in larger trials including people with Long Covid for longer than 4 months and in more diverse populations, but this trial lends credence to the hypothesis that dysregulated microbiota play a role in Long Covid.

A few other small trials have been published, some with promising results and others with negative results. In a small but moderately successful double-blind clinical trial, 41 people with Long Covid were randomized to AXA1125 versus placebo twice daily for 4 weeks (*199*). AXA1125 is a combination of 5 amino acids and *N*-acetylcysteine that is thought to lead to improved mitochondrial function and decreased oxidative stress. Although 52% of participants taking AXA1125 reported adverse events, none were serious, and people in that group experienced significant improvements in fatigue-based symptoms compared with placebo. Another small randomized, double-blind trial of 73 people assigned to receive 40 daily treatments with hyperbaric oxygen (100% oxygen) versus sham found that those who received hyperbaric oxygen reported improvement in cognitive function, sleep, pain, and mood accompanied by improvement in perfusion and structural changes in brain MRI scans (*200*) and that these effects persisted for at least 1 year after treatment (*201*). Larger studies in more diverse populations are also needed to confirm and extend these positive trial results. Conversely, two double-blind controlled trials reported failures in alleviating Long Covid symptoms: one of the antioxidant coenzyme Q10 taken for 6 weeks (*202*) and another of prefrontal cortex active transcranial direct current stimulation (tDCS) versus sham tDCS (*203*).

Clinical trials of antivirals in people with already-established Long Covid test the hypothesis that persistently replicating SARS-CoV-2 is a cause of Long Covid. The first clinical trial of an antiviral to report results was the STOP-PASC trial, a single-center, double-blind randomized controlled trial in which 155 adults with Long Covid and at least two self-reported moderate-to-severe symptoms were randomized 2:1 to 15 days of oral nirmatrelvir-ritonavir (Paxlovid) or placebo-ritonavir (100 mg) twice daily (*204*). Although this trial demonstrated the safety of a 15-day course of nirmatrelvir-ritonavir in people with Long Covid, unfortunately, nirmatrelvir-ritonavir did not have a positive effect on Long Covid outcomes. However, the investigators observed that both treatment groups experienced improvements in symptoms during the 15-week follow-up period.

Although the results of the STOP-PASC trial may dampen enthusiasm for clinical trials of antivirals, there may be a role for further investigation of antivirals. An antiviral might only be effective in a subpopulation of people who harbor viral reservoirs. For example, in the STOP-PASC trial, no participant had detectable SARS-CoV-2 RNA in their stool before the intervention (*204*). It may also be the case that antivirals may be more effective at post-acute time points closer to the index infection or that longer durations of antivirals are required. One of the large, US NIH–sponsored RECOVER clinical trials (RECOVER-VITAL) is currently trialing antivirals for Long Covid and may be able to resolve these questions. In addition, several antiviral strategies are currently being trialed outside of RECOVER-VITAL, including other trials of nirmatrelvir/ritonavir (*205*–*207*), ensitrelvir (*208*), remdesivir (*209*), and anti–SARS-CoV-2 monoclonal antibodies (*210*).

In hopeful news, there are several large, well-designed clinical trials for Long Covid currently underway. The UK-based STIMULATE-ICP trial began enrollment in August 2022, aims to recruit more than 1200 people, and is trialing the anti-inflammatory medication colchicine, the anti-clotting medication rivaroxaban, and two antihistamines in combination (*211*). The NIH RECOVER clinical trial program has thus far opened enrollment in three large, multicenter platform clinical trials in 2023 and 2024, with plans to initiate others later in 2024. RECOVER-VITAL will trial antiviral therapies, currently placebo versus nirmatrelvir-ritonavir for 15 or 25 days, in up to 900 participants (*212*). RECOVER-NEURO's design is similar and seeks to enroll more than 300 adults with neurocognitive Long Covid for a 10-week intervention delivered remotely in five arms. The interventions include an interactive online brain training program and the same program paired with either a virtual cognitive rehabilitation program or tDCS delivered by headset at home (*213*). RECOVER-AUTONOMIC will trial intravenous immunoglobulin versus placebo, ivabradine to reduce heart rate versus placebo, and wearing a compression belt and eating a high-salt diet versus usual care in up to 380 participants with POTS related to Long Covid (*214*). All RECOVER clinical trials are designed to be flexible and adaptive, and each can incorporate new interventions in the future. Canada has similarly launched an adaptive platform randomized trial hoping to recruit more than 800 participants with Long Covid, named the Recovering from COVID-19 Lingering Symptoms Adaptive Integrative Medicine (RECLAIM) study (*215*). Current interventions include ibudilast [an anti-inflammatory that can cross the blood-brain barrier and suppress glial cell activation (*216*)] or pentoxifylline (an immunomodulatory and anti-inflammatory agent) versus placebo taken orally for 2 months with follow-up through 6 months. These studies are not a comprehensive list of national Long Covid trial initiatives, and it is hoped that other countries follow suit with large trials of Long Covid therapeutics.

Trials for many other agents for Long Covid are underway, as reviewed previously (*107*). Several agents that may reverse immune dysfunction are being trialed, including baricitinib (*217*), anakinra (*218*), sirolumus (*219*), methylprednisolone (*220*), and atorvastatin (*221*), among many others. For respiratory Long Covid, the asthma medication montelukast and S-1226, a mixture of 8% $CO_2$ in perflubron, are being trialed (*222*, *223*). For fibromyalgia-type Long Covid, sublingual cyclobenzaprine, a muscle relaxant, is being evaluated in a trial (*224*). Other agents such as lithium (*225*); the diabetes medication metformin (*226*); the antidepressant fluvoxamine (*226*, *227*); larazotide (*228*), which may reverse leaky gut walls; and many others are in current or planned clinical trials for Long Covid.

## CONCLUSIONS

The heterogeneity of symptoms and baseline characteristics of people with Long Covid have complicated attempts to determine the etiologies of this condition. Large human studies are needed to investigate the fundamental causes of persistent symptoms and to define disease mechanisms. In addition to the promise of current and planned clinical trials in ameliorating disease, the knowledge of which agents are and are not effective will also provide insight into the pathophysiology of Long Covid and highlight pathways that might be even more specifically targeted. The large number of people with Long Covid and its involvement in nearly every organ system of the body has resulted in an engaged community of researchers with a broad range of expertise motivated to make a difference. The application of knowledge and tools from diverse areas of science and medicine opens enormous potential to further our understanding and to complete transformative mechanistic and clinical studies. In advancing effective therapies for people with Long Covid, there is the potential to uncover shared mechanisms and therapeutics for myalgic encephalomyelitis/chronic fatigue syndrome and post-acute infectious syndromes caused by other pathogens.

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**Acknowledgments:** We thank L. Gutierrez for creating and revising figures, S. Warner for helpful discussions, and A. Jamal for assistance with references. **Funding:** A.A.R.A. and A.L.C. are supported by NIH grant U19AI159822-02S1, A.L.C. is supported by U54CA260492, and A.A.R.A. is supported by NIH grant K08AI143391. **Competing interests:** The authors declare that they have no competing interests.

Submitted 1 April 2024
Accepted 18 October 2024
Published 13 November 2024
10.1126/scitranslmed.ado2106

LONG COVID

# Sex differences and immune correlates of Long Covid development, symptom persistence, and resolution

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**Sex differences have been observed in acute coronavirus disease 2019 (COVID-19) and Long Covid (LC) outcomes, with greater disease severity and mortality during acute infection in males and greater proportions of females developing LC. We hypothesized that sex-specific immune dysregulation contributes to LC pathogenesis. To investigate the immunologic underpinnings of LC development and symptom persistence, we performed multiomic analyses on blood samples obtained during acute severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and 3 and 12 months after infection in a cohort of 45 participants who either developed LC or recovered. Several sex-specific immune pathways were associated with LC. Males who would later develop LC exhibited increases in transforming growth factor–β (TGF-β) signaling during acute infection, whereas females who would go on to develop LC had reduced *TGFB1* expression. Females who developed LC demonstrated increased expression of *XIST*, an RNA gene implicated in autoimmunity, during acute infection compared with females who recovered. Many immune features of LC were also conserved across sexes, such as alterations in monocyte phenotype and activation state. Nuclear factor κB (NF-κB) transcription factors were up-regulated in many cell types at acute and convalescent time points. Those with ongoing LC demonstrated reduced *ETS1* expression across lymphocyte subsets and elevated intracellular IL-4 in T cell subsets, suggesting that *ETS1* alterations may drive aberrantly elevated T helper cell 2–like responses in LC. Altogether, this study describes multiple innate and adaptive immune correlates of LC, some of which differ by sex, and offers insights toward the pursuit of tailored therapeutics.**

## INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the virus that causes coronavirus disease 19 (COVID-19), has thus far led to more than 775,000,000 reported COVID-19 cases and more than 7,000,000 deaths globally since its first reported case in December 2019 (*1*). In addition to the morbidity and mortality caused by acute disease, a substantial proportion of patients (approximately 5 to 10%) continue to suffer from symptoms for months to years after SARS-CoV-2 infection, a condition known as Long Covid (LC), which has been defined by the World Health Organization (WHO) as symptoms persisting beyond 12 weeks after infection (*2*). LC is a heterogeneous condition with potential clinical signs and symptoms spanning nearly every organ system. These commonly include fatigue, myalgias, dyspnea, cough, chest pain, neurocognitive dysfunction, dysosmia, and dysgeusia (*2*). Given the heterogeneity of clinical presentations, LC may encompass potential subgroups of patients afflicted by different symptomatology and pathophysiology.

There are many proposed, not mutually exclusive, pathophysiologic mechanisms for LC. These include immune dysregulation (*3*–*7*), SARS-CoV-2 persistence (*8*, *9*), SARS-CoV-2–specific (*3*, *6*) and auto-reactive antibody responses (*4*, *10*), human herpesvirus [e.g., Epstein-Barr virus (EBV)] reactivation (*11*), microvascular and clotting dysfunction (*12*), thromboinflammation (*13*), amyloid deposition (*12*, *14*), mitochondrial dysfunction (*14*), dysbiosis (*15*), and hormonal or metabolic abnormalities, such as cortisol (*3*, *4*) or serotonin (*16*) dysregulation. Prior studies on immune dysregulation have implicated different immune cell populations in the pathophysiology of LC, but a unifying etiology across different studies has not been discovered (*17*).

Sex-based differences have underpinned outcomes for both acute COVID-19 and LC. Within the first year of the pandemic, males were observed to have higher rates of critical illness and death from acute COVID-19 compared with females (*18*). Conversely, female sex has been epidemiologically associated with increased risk of developing LC, with a meta-analysis reporting a pooled estimated odds ratio of 1.57 for females developing LC compared with males (*19*). Females are also more likely to experience cardiopulmonary and constitutional LC symptoms (*20*). Although the exact mechanisms underlying these sex differences remain to be fully elucidated, several innate and adaptive immunologic differences have been noted between the sexes (*21*, *22*). After symptomatic COVID-19, females were found to have higher percentages of $CD19^+$ B cells and $CD4^+$ T cells compared with males, whereas males were noted to have increased concentrations of inflammatory markers such as C-reactive protein and interleukin-6 (IL-6) compared with females (*23*). Males were also found to have slower SARS-CoV-2 viral clearance than females (*24*). Although there is a similarity between the higher prevalence of females with LC and autoimmune conditions, such as systemic lupus erythematosus (SLE), Sjogren's syndrome,

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and systemic sclerosis (*21*, *22*, *25*), the mechanisms underlying sex differences in LC require further investigation.

We hypothesized that sex-specific, dysregulated immune cell communication during both acute COVID-19 and convalescence contributes to the pathogenesis of LC. To investigate this hypothesis, we performed comprehensive multiomic profiling in 45 participants from a cohort of patients who were recruited at 3 months after infection to study recovery from SARS-CoV-2 infection. Many participants had ongoing LC symptoms at 3 and 12 months after infection, affording us the opportunity to evaluate predictors of LC versus recovery. We performed single-cell RNA-sequencing (scRNA-seq) and proteomic analyses, focusing our analyses on networks of gene activation and inferred cell-cell communication pathways to gain insights into the pathways associated with LC.

## RESULTS

### Cohort description

Multiple assays were performed on samples from 45 participants from the prospective, observational cohort called "Infection Recovery in SARS-CoV-2" (IRIS) at Stanford University to evaluate immune function during recovery from COVID-19. Given a likely selection bias among participants with LC symptoms to enroll in the IRIS cohort, individuals who underwent immune profiling for this analysis were enriched for LC diagnosis. Demographics of the cohort as assessed at the enrollment visit at 3 months after infection are presented in Table 1.

At 3 months after infection, 9 participants had fully recovered and 36 had persistent symptoms indicative of LC. Of the nine participants who recovered, 44.4% were female, the mean age was 41.7 years, and one participant (11.1%) was previously hospitalized for acute SARS-CoV-2 infection (Table 1). Of the 36 participants who developed LC at 3 months after infection, 55.6% were female, the mean age was 43.0 years, and 8 participants (22.2%) were previously hospitalized for acute SARS-CoV-2 infection (Table 1). Only one participant was taking exogenous sex hormone therapy (norethindrone and ethinyl estradiol), and one participant received inhaled corticosteroid (budesonide) during the study. Most participants received a COVID-19 vaccine during the study period, but no vaccine doses were administered before the 3-month study visit (table S1). A variety of symptoms were present among both male and female participants, with constitutional, cognitive, and pulmonary symptoms being most prevalent (Fig. 1A).

Banked peripheral blood mononuclear cell (PBMC) samples from these participants during acute infection, at 3 months after infection, and at 12 months after infection were used for scRNA-seq and cytometry by time-of-flight (CyTOF) (Fig. 1B). Olink proteomics was performed on plasma samples (Fig. 1B). For scRNA-seq data, cell types were annotated with Seurat v4 PBMC multimodal reference mapping (*26*) to identify multiple innate and adaptive immune cell types. Within individual cell types, we assessed expression changes in gene modules and inferred ligand-receptor interaction analyses with target gene predictions (Fig. 1C). CyTOF and plasma proteomic data were used to validate findings from transcriptional analyses.

### Blood transcription modules during acute SARS-CoV-2 infection differed on the basis of sex

To identify immune dysregulation during acute infection that might predict LC, we performed transcriptional and proteomic analyses on the 21 participants (of the original 45) who had samples available during acute infection (table S2). Among these 21 individuals, 15 participants went on to develop LC at 3 months after infection, and 6 participants fully recovered by 3 months after infection (table S2). Of the six participants who went on to recover, 50% were female, the mean age was 41.8 years, and none were hospitalized for acute SARS-CoV-2 infection (table S2). Of the 15 participants who developed LC, 60% were female, the mean age was 45.8 years, and 1 participant (6.7%) was hospitalized for acute SARS-CoV-2 infection (table S2).

We first sought to identify transcriptional predictors of the development of LC using blood transcription modules (BTMs) sourced from a reference gene set database dedicated to studying blood transcriptional immune responses (*27*). Numerous BTMs differed during acute infection between participants who would later develop LC and those who recovered by 3 months after infection. The "type I interferon response" module, which is critical for antiviral immune responses, was increased in acute samples of participants who developed LC in monocyte, B cell, and T cell subsets (Fig. 1D). The "chromosome Y linked" module was decreased in participants who would develop LC across many cell types (Fig. 1D and fig. S1A). The genes driving this BTM difference were *X-inactive specific transcript* (*XIST*), *DEAD-Box helicase 3 Y-Linked* (*DDX3Y*), *ubiquitously transcribed tetratricopeptide repeat containing, Y-Linked* (*UTY*/*KDM6C*), *lysine demethylase 5D* (*KDM5D*), *protein kinase Y-Linked* (*PRKY*), and *ubiquitin specific peptidase 9 Y-Linked* (*USP9Y*), which are sex-linked genes thought to have important roles in immune responses (*28*–*33*) (table S3). A larger proportion of females than males in the cohort progressed to LC after acute infection, although this difference was not significant (75% versus 67%, $P > 0.2$). The larger proportion of females in the LC cohort may account for a portion of these sex-linked gene expression differences when the sexes are analyzed together.

On the basis of these findings and the evidence for sex-related differences in COVID-19 outcomes (*18*, *19*), we next analyzed the sexes separately (Fig. 1E). When gene expression differences were separated by sex, expression of multiple sex-related genes, including *DDX3Y*, *UTY*, *PRKY*, and *USP9Y*, differed in males who progressed to LC compared with those who recovered (fig. S1B). *DDX3Y*, the Y-linked homolog of *DEAD-Box helicase 3 X-Linked* (*DDX3X*) with known roles in the interferon (IFN) response to pathogens (*29*), was down-regulated across most cell subsets at 12 months in males with LC (fig. S1B). In $CD14^+$ monocytes, conventional dendritic cells 2 (cDC2s), and $CD8^+$ T effector memory ($T_{EM}$) cells, these findings were statistically significant (adjusted $P < 0.05$) at both 3 and 12 months after acute infection (fig. S1C to E). The RNA gene *XIST*, which has previously been implicated in autoimmunity (*28*), was more highly expressed in several innate and adaptive immune cell subsets during acute infection in females who subsequently developed LC (fig. S1F). At 3 and 12 months after acute infection, increases in *XIST* in females with LC were still present but less prominent (fig. S1F). Sex hormones, such as estrogens and androgens, were not measured in this study. However, we evaluated pseudobulk expression of major sex hormone receptor genes *estrogen receptor 1* (*ESR1*), *estrogen receptor 2* (*ESR2*), and *androgen receptor* (*AR*) and did not identify pervasive differences between LC and recovered groups (fig. S1G and H).

Beyond sex-related genes, analyzing the sexes separately revealed differences between males and females in the acute infection

**Table 1. Demographics of the overall cohort at 3 months after acute SARS-CoV-2 infection, separated by LC versus recovered symptom status at 3 months.**

| | | | | Female | | Male | |
|---|---|---|---|---|---|---|---|
| | Total | Recovered | Long Covid | Recovered | Long Covid | Recovered | Long Covid |
| | (*N* = 45) | (*N* = 9) | (*N* = 36) | (*N* = 4) | (*N* = 20) | (*N* = 5) | (*N* = 16) |
| *Sex* | | | | | | | |
| Female | 24 (53.3%) | 4 (44.4%) | 20 (55.6%) | 4 (100%) | 20 (100%) | 0 (0%) | 0 (0%) |
| Male | 21 (46.7%) | 5 (55.6%) | 16 (44.4%) | 0 (0%) | 0 (0%) | 5 (100%) | 16 (100%) |
| *Age (years)* | | | | | | | |
| Mean (SD) | 42.8 (12.3) | 41.7 (9.45) | 43.0 (13.0) | 41.8 (10.4) | 41.2 (12.2) | 41.6 (9.89) | 45.3 (14.1) |
| Median [min, max] | 43.0 [20.0, 67.0] | 42.0 [27.0, 53.0] | 43.0 [20.0, 67.0] | 45.5 [27.0, 49.0] | 40.0 [20.0, 63.0] | 41.0 [27.0, 53.0] | 43.0 [26.0, 67.0] |
| *Race* | | | | | | | |
| White | 36 (80.0%) | 5 (55.6%) | 31 (86.1%) | 1 (25.0%) | 19 (95.0%) | 4 (80.0%) | 12 (75.0%) |
| Asian | 7 (15.6%) | 3 (33.3%) | 4 (11.1%) | 2 (50.0%) | 1 (5.0%) | 1 (20.0%) | 3 (18.8%) |
| Black or African American | 1 (2.2%) | 0 (0%) | 1 (2.8%) | 0 (0%) | 0 (0%) | 0 (0%) | 1 (6.3%) |
| American Indian | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Pacific Islander | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Other | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Missing | 1 (2.2%) | 1 (11.1%) | 0 (0%) | 1 (25.0%) | 0 (0%) | 0 (0%) | 0 (0%) |
| *Ethnicity* | | | | | | | |
| Not Hispanic or Latino | 27 (60.0%) | 8 (88.9%) | 19 (52.8%) | 4 (100%) | 8 (40.0%) | 4 (80.0%) | 11 (68.8%) |
| Hispanic or Latino | 18 (40.0%) | 1 (11.1%) | 17 (47.2%) | 0 (0%) | 12 (60.0%) | 1 (20.0%) | 5 (31.3%) |
| *Body mass index* | | | | | | | |
| Mean (SD) | 30.2 (5.87) | 31.3 (7.86) | 30.0 (5.37) | 34.0 (10.1) | 31.1 (6.12) | 29.0 (5.78) | 28.7 (4.04) |
| Median [min, max] | 29.0 [19.6, 45.1] | 27.8 [22.4, 44.6] | 29.4 [19.6, 45.1] | 34.1 [23.3, 44.6] | 30.4 [19.6, 45.1] | 27.2 [22.4, 38.0] | 28.3 [22.9, 36.9] |
| *Hospitalized* | | | | | | | |
| No | 36 (80.0%) | 8 (88.9%) | 28 (77.8%) | 4 (100%) | 17 (85.0%) | 4 (80.0%) | 11 (68.8%) |
| Yes | 9 (20.0%) | 1 (11.1%) | 8 (22.2%) | 0 (0%) | 3 (15.0%) | 1 (20.0%) | 5 (31.3%) |
| *Intensive care unit* | | | | | | | |
| No | 41 (91.1%) | 8 (88.9%) | 33 (91.7%) | 4 (100%) | 19 (95.0%) | 4 (80.0%) | 14 (87.5%) |
| Yes | 4 (8.9%) | 1 (11.1%) | 3 (8.3%) | 0 (0%) | 1 (5.0%) | 1 (20.0%) | 2 (12.5%) |
| *Intubated* | | | | | | | |
| No | 44 (97.8%) | 9 (100%) | 35 (97.2%) | 4 (100%) | 19 (95.0%) | 5 (100%) | 16 (100%) |
| Yes | 1 (2.2%) | 0 (0%) | 1 (2.8%) | 0 (0%) | 1 (5.0%) | 0 (0%) | 0 (0%) |
| *Treatment during acute COVID-19* | | | | | | | |
| Remdesivir | 8 (17.8%) | 1 (11.1%) | 7 (19.4%) | 0 (0%) | 2 (10.0%) | 1 (20.0%) | 5 (31.3%) |
| Peginterferon lambda-1a | 13 (28.9%) | 2 (22.2%) | 11 (30.6%) | 1 (25.0%) | 5 (25.0%) | 1 (20.0%) | 6 (37.5%) |
| Favipiravir | 8 (17.8%) | 3 (33.3%) | 5 (13.9%) | 3 (75.0%) | 3 (15.0%) | 0 (0%) | 2 (12.5%) |
| None or placebo | 16 (35.6%) | 3 (33.3%) | 13 (36.1%) | 0 (0%) | 10 (50.0%) | 3 (60.0%) | 3 (18.8%) |

gene profiles of those who went on to develop LC versus those who recovered. There were prominent gene expression differences in multiple BTMs in proliferating natural killer (NK) cells and reduced expression in the "leukocyte activation and migration" module for several T cell subsets in males who developed LC (Fig. 1F). In contrast, females who developed LC had expression changes that were less restricted to particular cell types or modules, affecting multiple lymphoid and myeloid subsets (Fig. 1F). Increased expression of BTMs in $CD14^+$ and $CD16^+$ monocytes was found in both sexes of those who developed LC (Fig. 1F). Given these sex-specific differences found in BTM analyses of acutely infected samples, subsequent analyses of these samples focused on proliferating NK cells, monocytes, and sex-specific differences in several key regulators of inflammation.

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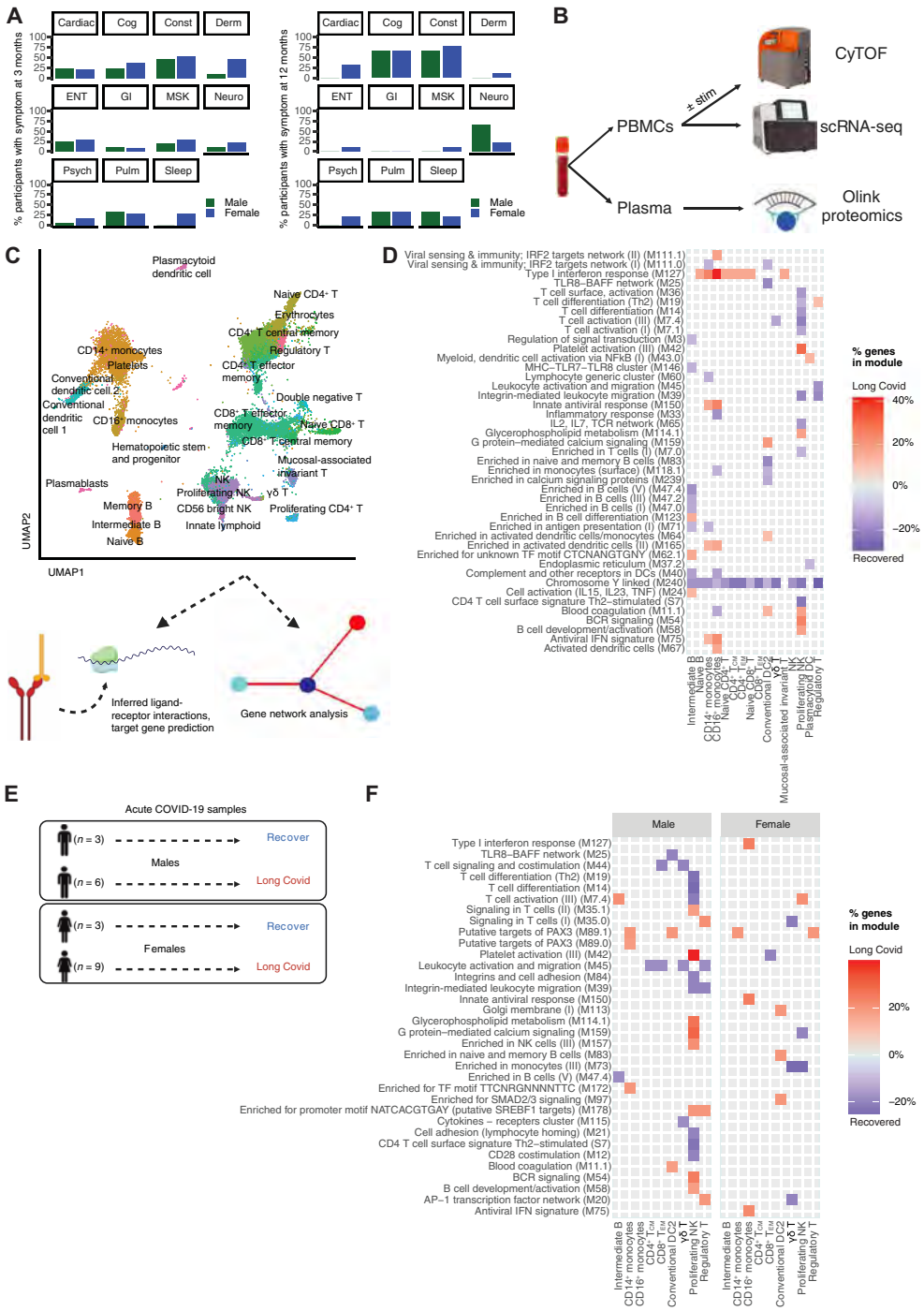



**Fig. 1. BTM differences during acute infection were observed between those who recovered and those who developed LC.** (**A**) Symptom prevalence of participants with LC at 3 months after acute infection ($n = 36$) and 12 months after acute infection ($n = 12$). Cog, cognitive; Const, constitutional; Derm, dermatologic; ENT, ear, nose, and throat; GI, gastrointestinal; MSK, musculoskeletal; Neuro, neurologic; Psych, psychiatric; Pulm, pulmonary; Sleep, sleep disturbance. (**B**) Experimental design demonstrating use of CyTOF and scRNA-seq for PBMC samples and Olink proteomics for plasma samples. (**C**) UMAP of scRNA-seq data (subsampled to 100,000 cells to reduce overplotting), with individual cell types further analyzed with MultiNicheNet for cell-cell communication inference and BioNet for gene network analysis. (**D**) BTMs of samples during acute infection that differentiated individuals with LC versus those who recovered by 3 months after infection for males and females combined. Only modules in which >10% of the constituent genes had an absolute log2 fold change (log2FC) >0.25 are plotted. Red tiles indicate higher expression in individuals who developed LC, and blue tiles indicate lower expression. (**E**) Participants included in acute SARS-CoV-2 infection analysis, separated by sex and LC versus recovered status at 3 months after infection. (**F**) BTMs during acute infection that differentiated those with LC versus those who recovered by 3 months after infection. Males and females are plotted separately. The top 50 modules, by percentage of genes, in which >10% of the constituent genes had an absolute log2FC >0.25, are plotted. Red tiles indicate higher expression in individuals who developed LC, and blue tiles indicate lower expression.

### Acute *TGFB1* signaling in proliferating NK cells is associated with LC development in males

To understand the drivers of the transcriptional changes observed in BTM analysis (Fig. 1F), we used MultiNicheNet to infer communication between proliferating NK cells and other immune cells during acute infection (*34*). Most of the top ligand-receptor interactions with proliferating NK cells as senders of signal in acutely infected male samples were dominated by genes associated with transforming growth factor–β1 (TGF-β1) signaling, as well as genes associated with interferon-γ (IFN-γ) signaling, in those who would develop LC at 3 months after infection compared with those who would recover (Fig. 2A).

Similarly, most of the top interactions received by proliferating NK cells were dominated by TGF-β1 signaling from several NK cell and T cell populations in acute samples from males who would develop LC at 3 months (Fig. 2B). Individual participant data for *TGFB1* and *TGFBR1* are shown in Fig. 2 (C and D). Cell-cell interactions toward proliferating NK cells were correlated with the up-regulation of the chemokine C-C motif chemokine ligand 3 (*CCL3*), which can augment NK cell cytolytic activity and can be produced by activated NK cells to recruit other effector cells (*35*); death associated protein kinase 1 (*DAPK1*), which is involved in IFN-γ–induced apoptosis and may promote NK cell killing (*36*, *37*); IFN-induced protein with tetratricopeptide repeats 2 (*IFIT2*), which can promote apoptosis (*38*); *ZNF395*, which can activate IFN-stimulated genes (*39*); the protease furin (*FURIN*), which is also a TGF-β1–converting enzyme (*40*); insulin-like growth factor binding protein 7 (*IGFBP7*), which can up-regulate TGF-β1 signaling (*41*); and polypeptide *N*-acetylgalactosaminyltransferase 10 (*GALNT10*), which may be associated with an immunosuppressive phenotype (*42*); among others (Fig. 2E). This TGF-β1 signaling was also predicted to down-regulate ADAM metallopeptidase with thrombospondin type 1 motif 6 (*ADAMTS6*), which can increase TGF-β1 activation (*43*); SMAD family member 3 (*SMAD3*), which is an intracellular signaling component of the TGF-β pathway but has previously been shown to be down-regulated in response to TGF-β in certain contexts (*44*); TNF receptor–associated protein 1 (*TRAP1*), which is suggested to have cytoprotective effects against oxidative stress and reactive oxygen species (*45*, *46*); and ETS proto-oncogene 1 (*ETS1*), which regulates NK cell development and terminal differentiation (*47*); among others (Fig. 2E). Although TGF-β1 is best described as an inhibitory signal in NK cells, these transcriptional differences also suggest that cytolytic functions and antiviral responses may be altered by TGF-β1 signaling in this context.

In addition to TGF-β1 signaling, MultiNicheNet also showed differences in interleukin-7 (IL-7) signaling in acutely infected males who went on to develop LC, which is important for NK cell survival and cytotoxicity (*48*) (Fig. 2B). This was predicted to result in down-regulation of interleukin-7 receptor (*IL7R*), suggesting a negative regulatory mechanism (Fig. 2E) (*49*). Pseudobulk *IL7R* expression during acute infection was down-regulated in males who developed LC in most cell types, including proliferating NK cells, and this pattern was not observed in females (Fig. 2F). The predicted effects on downstream target genes in proliferating NK cells were also apparent and consistent across individuals (fig. S2).

Unlike in males, cell-cell communication analyses of proliferating NK cells in acutely infected females who went on to develop LC demonstrated signaling predominantly involving C-C motif chemokine ligand 5 (*CCL5*), CD99 molecule (*CD99*), ADAM metallopeptidase domain 10 (*ADAM10*), interleukin-15 (*IL15*), and neuregulin 1 (*NRG1*), among others, but did not demonstrate predominant TGF-β1 signaling (fig. S3). Given the prominent TGF-β1 signaling in proliferating NK cells of the acutely infected males who subsequently developed LC, we next examined whether TGF-β1 was important across different cell types and time points.

### *TGFB1* expression and protein secretion differs in individuals who develop LC in a sex-specific manner

During acute infection, *TGFB1* expression was increased in males who would develop LC not only in proliferating NK cells, but also in most innate and adaptive cell types (Fig. 3A). On the other hand, *TGFB1* expression was decreased during acute infection in females who would develop LC in most examined cell types (Fig. 3A). Differential expression analysis revealed significant (adjusted $P < 0.05$) reductions in *TGFB1* in NK, $CD8^+$ $T_{EM}$, and $CD4^+$ central memory T ($T_{CM}$) cells of females who went on to develop LC (Fig. 3B to D). There was no clear difference observed in the individual who received experimental antiviral therapy (fig. S4A to C) or hospitalization status (fig. S4D to F) during acute COVID-19. Corroborating the transcriptional findings with protein data, latency-associated peptide (LAP) TGF-β1 was significantly increased (unadjusted $P < 0.05$) in the plasma of acutely infected males who would develop LC compared with males who would recover; this difference was not observed in females (Fig. 3E).

We next evaluated *TGFB1* transcriptional and proteomic differences in all study participants at 3 months after infection between 36 individuals who developed LC versus 9 individuals who recovered (Table 1), with the analysis divided by sex (fig. S5A). Similar to the findings during acute infection, *TGFB1* expression was increased in males with LC in most examined cell types, whereas *TGFB1* expression was mostly decreased in the different cell types in females with LC (fig. S5A). The plasma protein LAP TGF-β1 was not significantly different in either sex ($P > 0.2$ for males and females) (fig. S5B).

In addition, a subgroup of the total 45 participants had samples available at both 3 and 12 months after infection to enable evaluation for ongoing LC (table S4). This analysis involved 12 participants who had ongoing LC at both 3 and 12 months after infection and 11 participants who had LC at 3 months but recovered at 12 months after infection. Of the 12 participants with ongoing LC at 3 and 12 months, 75% were female, the mean age was 44.8 years, and 1 participant (8.3%) was previously hospitalized for acute SARS-CoV-2 infection (table S4). Of the 11 participants who recovered at 12 months, 45.5% were female, the mean age was 37.3 years, and 3 participants (27.3%) were previously hospitalized for acute SARS-CoV-2 infection (table S4). Symptoms for this group of participants with LC at 12 months included increased prevalence of cognitive, constitutional, neurologic, and pulmonary symptoms (Fig. 1A). We evaluated the LC versus recovered groups separately by sex.

*TGFB1* expression was again found to be increased in males with ongoing LC at 12 months after infection in most cell types (fig. S5C). Unlike earlier time points, *TGFB1* expression was also increased in females with ongoing LC at 12 months after infection in most cell types (fig. S5C), although no difference in secreted LAP TGF-β1 in plasma was observed for either sex ($P > 0.2$ for males and $P = 0.11$ for females) (fig. S5D). Altogether, these data suggest that sex-specific differences in transcriptional and proteomic TGF-β1 are predictive of LC development, but these differences in TGF-β1 wane over time.

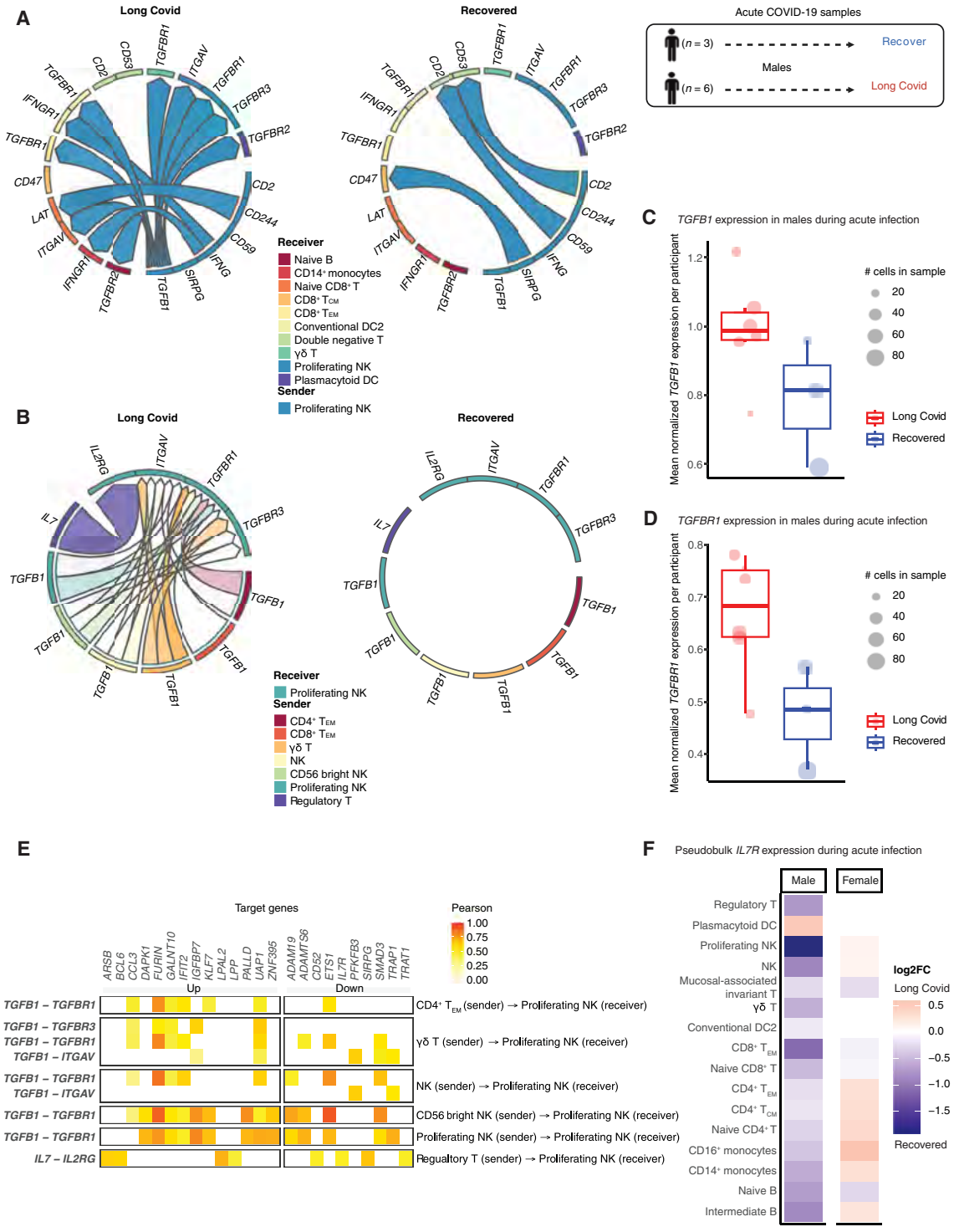



**Fig. 2. Proliferating NK cells exhibited different transcriptional profiles in males during acute infection based on LC status.** (**A** and **B**) Predicted ligand-receptor interactions involving proliferating NK cells, separated by development of LC symptoms 3 months after acute infection versus resolution of symptoms by 3 months, showing signaling sent from proliferating NK cells (A) and signaling received by proliferating NK cells (B). Arrows point from ligand to receptor. (**C** and **D**) Boxplots demonstrating mean normalized expression of *TGFB1* (C) and *TGFBR1* (D) during acute infection in males. (**E**) Target genes with altered expression in proliferating NK cells of acutely infected males who go on to develop LC at 3 months are shown with associated upstream ligand-receptor interactions. Tile color in the heatmap indicates the Pearson correlation coefficient of expression of the ligand-receptor pair and the target gene. Cell-cell communication analyses were performed with MultiNicheNet, which uses pseudobulk aggregation followed by edgeR analysis. (**F**) Pseudobulk *IL7R* expression by cell type during acute infection, separated by sex. Tile color in the heatmap indicates log2FC, with higher expression in those who will develop LC at 3 months after infection indicated by red and lower expression indicated by blue.

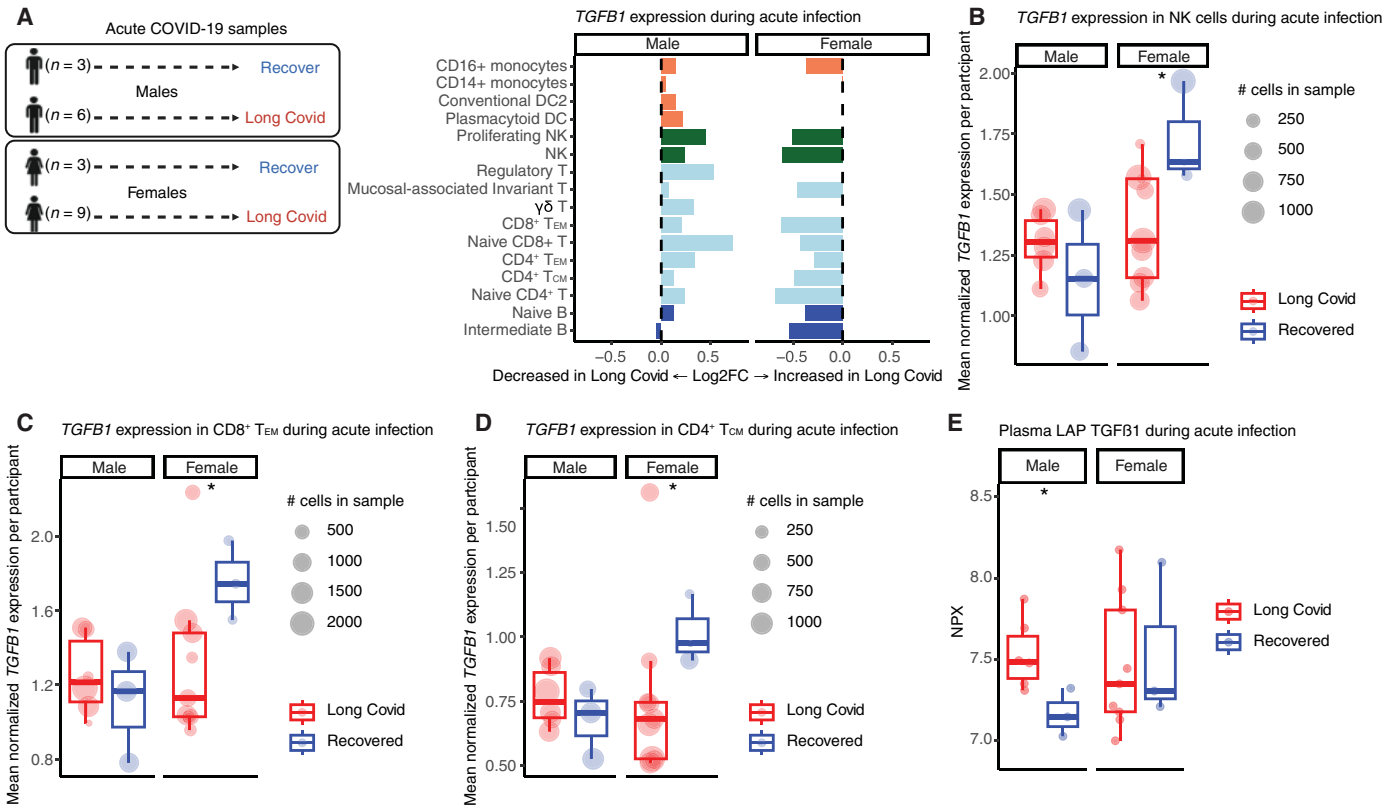



**Fig. 3. *TGFB1* expression differs by sex in those who develop LC.** (**A**) Log2FC of pseudobulk expression of *TGFB1* gene during acute infection, separated by sex. Bars to the right of the dashed line indicate increased expression and bars to the left indicate reduced expression in those who will develop LC at 3 months after infection. Myeloid cells are represented by orange bars, NK cells by green bars, T cells by light blue bars, and B cells by dark blue bars. (**B** to **D**) Boxplots of mean expression of *TGFB1* by participant during acute infection shows reductions in females who develop LC in NK cells (B), $CD8^+$ $T_{EM}$ cells (C), and $CD4^+$ $T_{CM}$ cells (D). * indicates locally adjusted $P < 0.05$ for differential gene expression by MAST, comparing those who go on to develop LC versus those who were recovered by 3 months. (**E**) Boxplot of normalized protein expression (NPX) of LAP TGF-β1 in plasma during acute infection, separated by sex. * indicates unadjusted $P < 0.05$ by Wilcoxon rank sum test between those who will go on to develop LC versus recover at 3 months after infection.

## Sex-specific signaling in monocytes during acute infection is associated with development of LC

Because of the differences of BTMs in monocytes during acute infection (Fig. 1, D and F), we further investigated these cells in both sexes with cell-cell communication and gene network analyses. In males, inference of ligand-receptor signaling toward monocytes showed that CD40 molecule (*CD40*) ligand signaling was most different during acute infection in those who went on to develop LC versus those who recovered at 3 months (Fig. 4A). $CD14^+$ monocytes and $CD16^+$ monocytes of participants who would recover received CD40 ligand (CD40LG) signaling from T cells, targeting multiple integrins that facilitate CD40 signaling (*50*–*52*), an important stimulus for inflammatory responses in infection and expression of costimulatory signals (*53*, *54*). $CD14^+$ monocytes in males who developed LC received pro-inflammatory *IFNG* and *IL15* signaling (*55*, *56*) as well as proapoptotic TNF superfamily member 10 (*TNFSF10*, also known as *TRAIL*) signaling (*57*) (Fig. 4A). $CD16^+$ monocytes in males who developed LC received CD226 molecule (*CD226*) signaling from NK and T cells directed to stimulate PVR cell adhesion molecule (*PVR*) and nectin cell adhesion molecule 2 (*NECTIN2*) (Fig. 4B). *PVR* encodes CD155, an inhibitory protein that has previously been associated with poor antiviral responses to SARS-CoV-2 (*58*). In addition, *PVR* and *NECTIN2* both promote tissue infiltration of monocytes (*59*), which has been associated with deleterious local inflammation (*60*). Thus, during acute infection, monocytes in males who will recover received CD40 ligand signaling that may promote costimulatory signals, whereas monocytes in males who will develop LC may be more prone to tissue infiltration.

In females, $CD14^+$ monocytes during acute infection in those who went on to develop LC received increased CCL5 signaling from NK and T cell subsets, and the monocytes themselves show increased expression of chemokine receptors C-C motif chemokine receptor 1 (*CCR1*) and C-C motif chemokine receptor 1 (*CCR3*) (Fig. 4C), a combination of which would be expected to promote chemotaxis. Higher expression of *CCR* genes in monocytes and macrophages has been associated with increased acute disease severity in COVID-19 (*61*). Gene network analysis for $CD14^+$ monocytes in females shows a subnetwork of IFN-stimulated and antiviral genes such as *MX dynamin like GTPase 1* (*MX1*), *MX dynamin like GTPase 2* (*MX2*), *tripartite motif containing 22* (*TRIM22*), *2′-5′-oligoadenylate synthetase 2* (*OAS2*), *2′-5′-oligoadenylate synthetase 3* (*OAS3*), *interferon induced protein 44* (*IFI44*), *DExD/H-Box 60 like* (*DDX60L*), *epithelial stromal interaction 1* (*EPSTI1*), *poly(ADP-ribose) polymerase family member 14* (*PARP14*), and *IFN-induced protein with tetratricopeptide repeats 3* (*IFIT3*) (*62*–*69*) (Fig. 4D), which were up-regulated in those who developed LC.

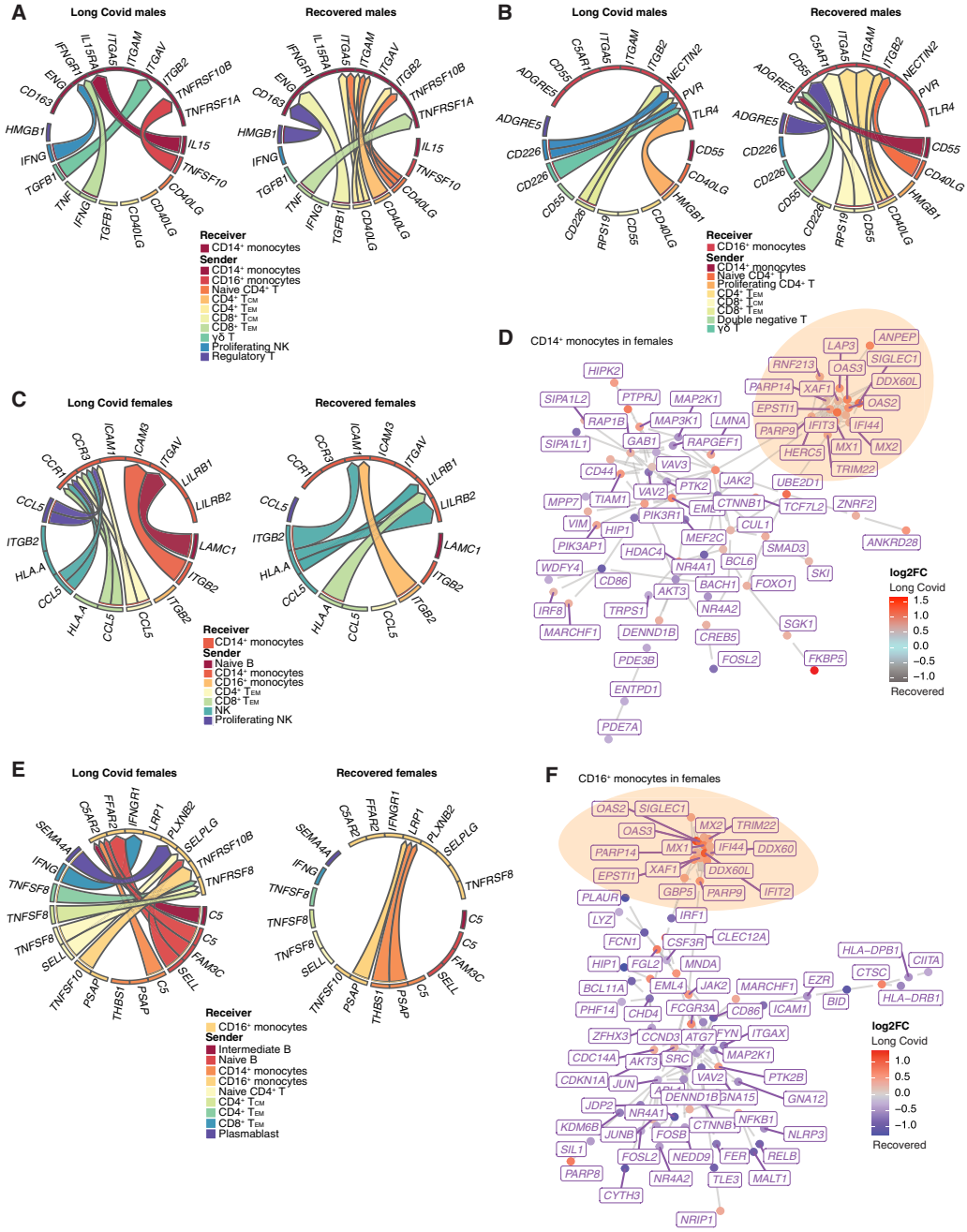



**Fig. 4. Sex-specific differences in inflammatory monocytes are present during acute SARS-CoV-2 infection.** (**A** and **B**) Inferred cell-cell communication, separated by development of LC symptoms 3 months after acute infection ($n = 6$) versus resolution of symptoms by 3 months ($n = 3$), toward CD14$^+$ monocytes (A) and CD16$^+$ monocytes (B) in males. (**C**) Cell-cell communication inference, separated by development of LC symptoms 3 months after acute infection ($n = 9$) versus resolution of symptoms by 3 months ($n = 3$), toward CD14$^+$ monocytes in females. Cell-cell communication analyses were performed with MultiNicheNet, which uses pseudobulk aggregation followed by edgeR analysis. (**D**) Differential gene network in CD14$^+$ monocytes in females during acute infection (false discovery rate $1 \times 10^{-5}$). Red nodes indicate higher expression (log2FC) in those who will develop LC, and blue nodes indicate lower expression. Light orange shading highlights an interferon-stimulated gene subnetwork. (**E**) Cell-cell communication inference, separated by development of LC symptoms 3 months after acute infection versus resolution of symptoms by 3 months, toward CD16$^+$ monocytes in females. (**F**) Differential gene network in CD16$^+$ monocytes in females during acute infection (false discovery rate $1 \times 10^{-10}$). Red nodes indicate higher expression (log2FC) in those who will develop LC, and blue nodes indicate lower expression. Light orange shading highlights an interferon-stimulated gene subnetwork.

$CD16^+$ monocytes in females who went on to develop LC received increased TNF signaling compared with those who recovered, including TNF superfamily member 8 (*TNFSF8*) (also known as *CD30L*) (Fig. 4E), which induces nuclear factor kappa B (NF-κB) activation and is implicated in autoimmunity (*70*). In addition, these monocytes received Complement 5 (*C5*) signaling, and complement dysregulation has previously been implicated in LC pathogenesis (*13*, *71*). Gene network analysis showed up-regulated IFN-stimulated and antiviral genes, including *PARP14*, *EPSTI1*, *OAS2*, *OAS3*, *MX1*, DExD/H-Box 60 (*DDX60*), *TRIM22*, *IFIT2*, and *IFI44* (*62*, *64*–*69*, *72*) (Fig. 4F). *IFI44*, which is up-regulated in gene networks for both $CD14^+$ and $CD16^+$ monocytes in females, has been observed to reduce IFN responses and has been associated with immune evasion in SARS-CoV-2 (*73*, *74*). In sum, transcriptional analyses of monocytes during acute infection revealed signatures suggestive of increased potential to infiltrate target tissues in individuals with LC, although the mechanisms differed by sex.

### Pro-inflammatory monocytes with reduced costimulatory expression are enriched in LC

We next turned our attention to the immune status at 3 and 12 months after infection. BTM analysis at 3 and 12 months in both males and females revealed ongoing differences in monocytes between participants with ongoing LC compared with those who recovered by 12 months (Fig. 5, A and B). Modules associated with chemokines, inflammatory molecules, and chemokine receptor signaling were up-regulated in $CD14^+$ monocytes at 3 months in those with ongoing LC (Fig. 5A). At 12 months after infection, males with ongoing LC showed up-regulation of numerous BTMs in $CD14^+$ monocytes, including modules associated with pro-inflammatory myeloid cell responses, chemokines, NF-κB activation, and antigen presentation, among others (Fig. 5B). BTM analysis of $CD16^+$ monocytes in females also showed up-regulation in BTMs associated with pro-inflammatory myeloid cell responses, NF-κB activation, and chemokines in those with ongoing LC at 12 months (Fig. 5B). $CD14^+$ monocytes in females with LC at 12 months also showed up-regulation of the module associated with chemokines and inflammatory molecules (Fig. 5B).

Cell-cell communication analysis of $CD14^+$ monocytes in males revealed much greater activity in individuals with LC at 12 months (fig. S6, A and B). Activating and pro-inflammatory signals such as CD58 molecule (*CD58*) and interleukin-1α (*IL1A*), which could interact with regulatory T cells, and S100 calcium binding protein A12 (*S100A12*), which could interact with $CD16^+$ monocytes and cDC2s, were enriched in those who have LC (fig. S6A). Conversely, interleukin-1 receptor antagonist (*IL1RN*; the anti-inflammatory antagonist of IL-1) (*75*) signaling toward cDC2s was also up-regulated in those with LC (fig. S6A). As receivers, $CD14^+$ monocytes in those with LC expressed genes associated with TGF-β1 signaling, which suggest interactions with NK cells and multiple T cell subsets (fig. S6B). These data are consistent with our finding that *TGFB1* transcription is up-regulated across all time points in males who developed LC (fig. S5C).

In females, different cell-cell communication patterns emerged. $CD14^+$ monocytes in those with LC at 12 months showed expression of genes associated with IL1 signaling toward memory B cells, cDC2s, and other $CD14^+$ monocytes (fig. S6C). These cells were also predicted to receive interleukin-1β (IL-1β) signals from $CD16^+$ monocytes, as well as TGF-β1 signals from NK cells, mucosal associated invariant T (MAIT) cells, gamma delta T cells (γδ T cells), and $CD8^+$ T cells (fig. S6D). These ligand-receptor interactions were predicted to increase expression of several genes that encode proteins involved in inflammation, such as C-X-C motif chemokine ligand 1 (*CXCL1*), C-X-C motif chemokine ligand 8 (*CXCL8*), interleukin-1β (*IL1B*), epiregulin (*EREG*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), and phorbol-12-myristate-13-acetate-induced protein 1 (*PMAIP1*) (fig. S6E).

Given the prevalence of IL-1β signaling in cell-cell communication analysis, we compared expression of this gene in monocytes in both sexes and found that *IL1B* expression was increased in $CD14^+$ monocytes in those with ongoing LC at 12 months after infection (adjusted $P < 0.05$) (Fig. 6A), although we did not observe significant up-regulation of interleukin-1α (IL-1α) protein in those with ongoing LC at 12 months ($P = 0.079$) (Fig. 6B). When we evaluated single-cell proteomics using CyTOF, individuals with LC persisting to 12 months had higher intracellular CXCL10 in unstimulated and stimulated $CD14^+$ monocytes ($P < 0.001$ and $P < 0.05$, respectively) compared with those without LC (Fig. 6, C and D). In stimulated samples, IL-1β was lower in LC, suggesting increased extracellular secretion of this protein in these individuals, given that it was transcriptionally up-regulated (Fig. 6D). Unstimulated $CD16^+$ monocytes of those with LC had higher C-X-C motif chemokine ligand 10 (CXCL10) and interleukin-6 (IL-6) ($P < 0.01$ and $P < 0.001$, respectively) at 12 months, whereas these differences were not observed in stimulated samples ($P = 0.16$ and $P > 0.2$, respectively) (Fig. 6, E and F). There were no clear differences in the participant who received antivirals or the individual who was hospitalized during acute infection (fig. S7).

In addition, $CD14^+$ monocytes had a set of genes with higher expression (adjusted $P < 0.05$) in males and females with LC across all time points. This gene set consists mostly of IFN-stimulated genes (ISGs), including *EPSTI1*, *guanylate binding protein 2* (*GBP2*), *HECT and RLD domain containing E3 ubiquitin protein ligase 5* (*HERC5*), *IFN-induced protein 44 like* (*IFI44L*), *IFIT3*, *leucine aminopeptidase 3* (*LAP3*), and *ubiquitin-conjugating enzyme E2 D1* (*UBE2D1*) (Fig. 6, G to M). Furthermore, *poly(ADP-ribose) polymerase family member 9* (*PARP9*), which detects RNA virus nucleic acids and activates type I IFN responses (*76*), was up-regulated across all time points (Fig. 6N). These findings were not clearly different in the individuals who received treatment or the participants who were hospitalized during acute infection (fig. S8).

We also found that $CD14^+$ monocytes of individuals with LC at 3 and 12 months had significantly reduced expression of CD86 molecule (*CD86*) (adjusted $P < 0.05$) (Fig. 6O), which is a costimulatory molecule expressed on antigen-presenting cells (*77*) that interacts with CD28 molecules on T cells to help induce proliferation and cytotoxicity (*78*). Plasma proteomics also supported reduced costimulatory function of monocytes, given that leukocyte immunoglobulin like receptor B4 (LILRB4), which has been shown to induce immune tolerance in antigen-presenting cells and an immunosuppressive T cell phenotype (*79*, *80*), was significantly higher ($P < 0.05$) in individuals with LC at 12 months (fig. S9A). In addition, several proteomic markers of T cell exhaustion, including interleukin-10 (IL-10) ($P < 0.05$), interleukin-10 receptor subunit beta (IL-10Rβ) ($P < 0.05$), and lymphocyte activating 3 (LAG3) ($P < 0.001$), were higher in those with ongoing LC at 12 months (fig. S9A). However, only LAG3 was significantly elevated ($P < 0.01$) in those with LC at 3 months who would have ongoing LC at 12

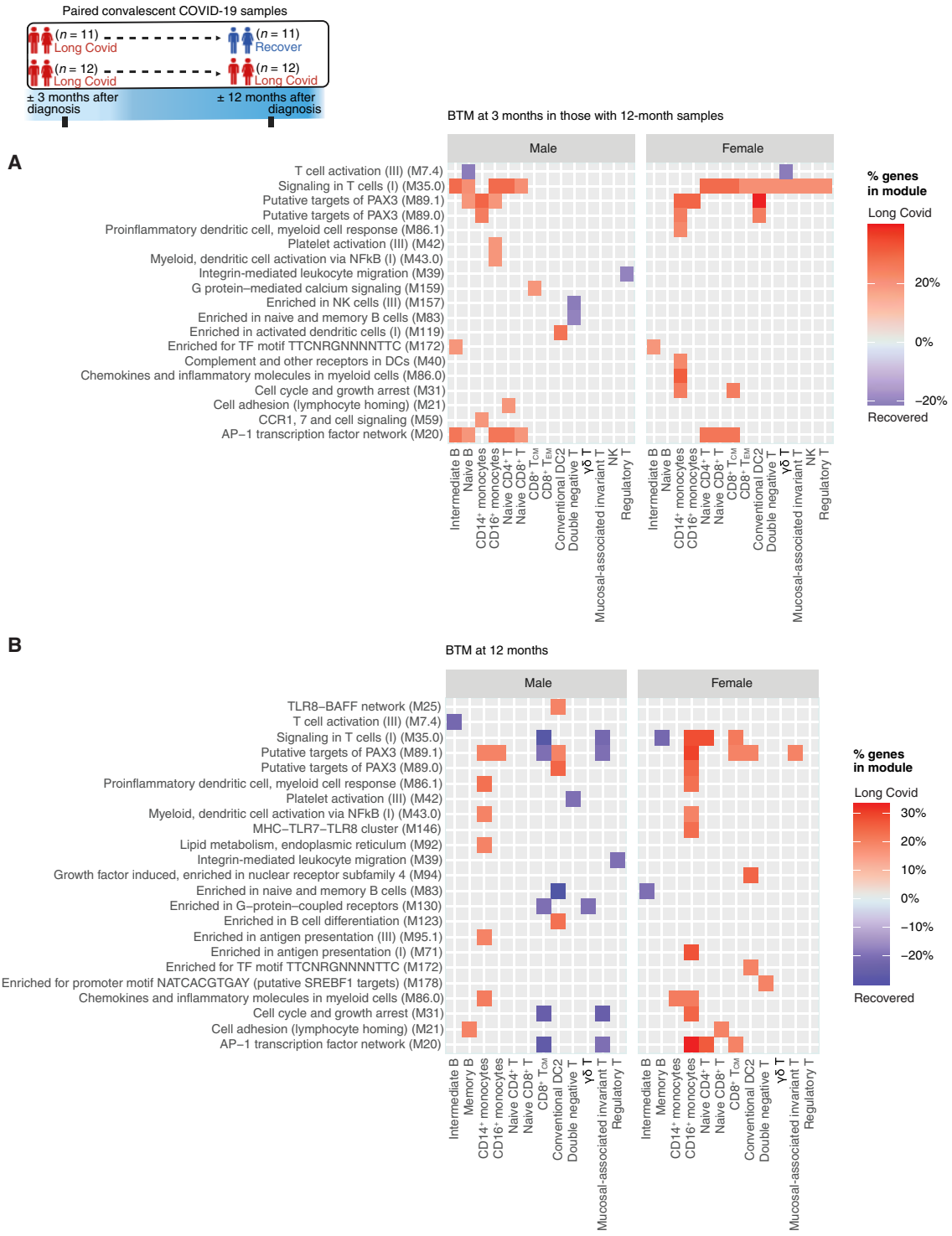



**Fig. 5. BTMs at 3 and 12 months differ between those who recover and individuals with LC.** (**A**) BTMs at 3 months after acute infection, separated by sex, which differentiate those with ongoing LC at 12 months after infection versus those who will recover by 12 months. (**B**) BTMs at 12 months after acute infection, separated by sex, which differentiate those with ongoing LC at 12 months after infection versus those who recovered between 3 and 12 months. The top 50 modules, by percentage of genes, in which >10% of the constituent genes had an absolute log2FC >0.25, are plotted. Red tiles indicate higher expression in individuals with LC, and blue tiles indicate lower expression.

months compared with those who would recover by 12 months (fig. S9B), and a T cell exhaustion gene score did not corroborate exhaustion of any T cell subsets (fig. S9, C and D). Altogether, monocytes of participants with ongoing LC demonstrated sex-specific differences in cell-cell communication analysis as well as some shared features.

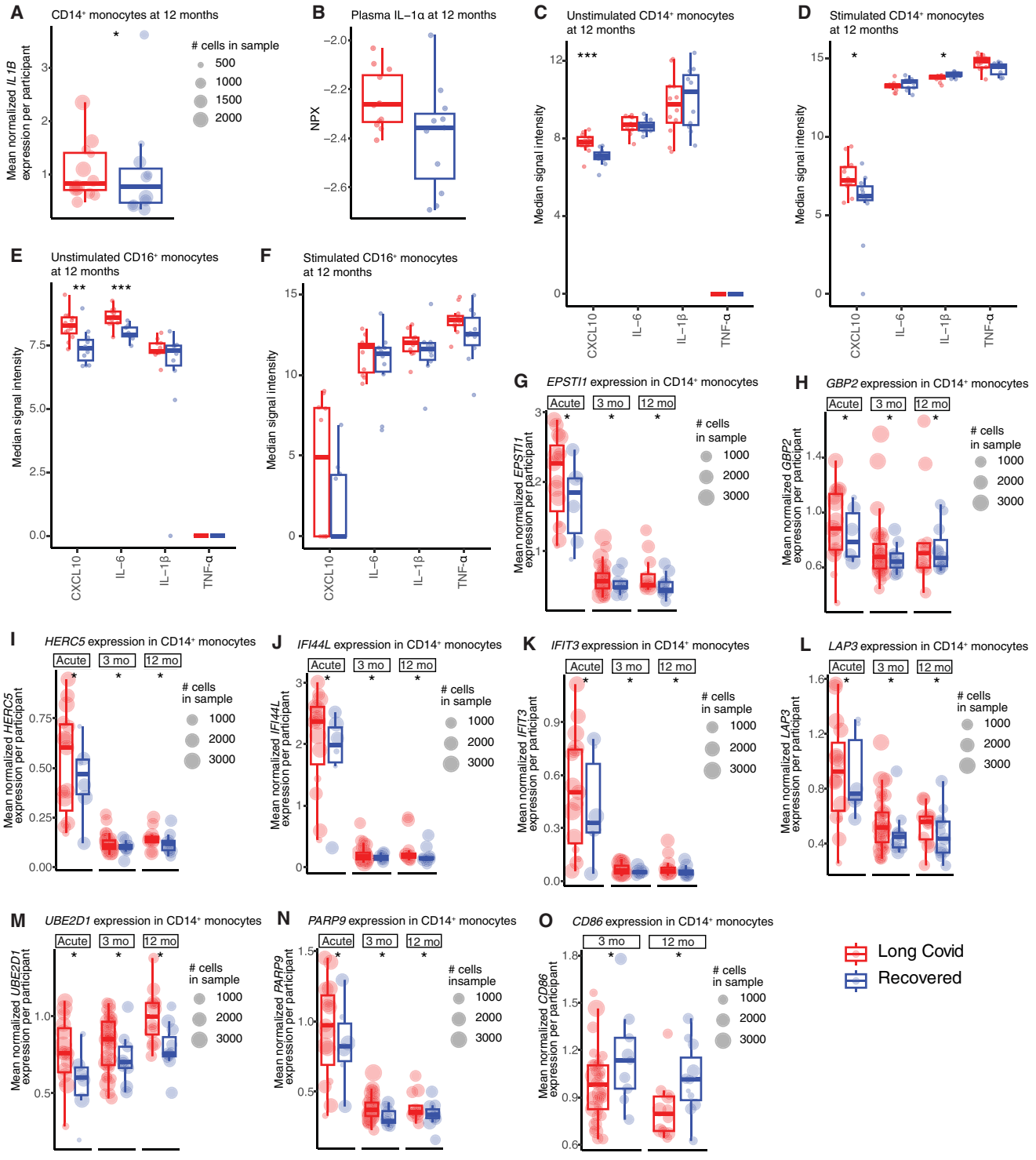



**Fig. 6. Inflammatory markers and ISG expression are increased in monocytes of individuals with ongoing LC.** (**A**) Boxplot of mean normalized *IL1B* expression for each participant is shown for CD14$^+$ monocytes of those with LC at 12 months after acute infection. (**B**) Boxplot of IL-1α abundance is shown for those with LC at 12 months by plasma proteomics. (**C** to **F**) Boxplots of median signal intensity by CyTOF of intracellular CXCL10, IL-6, IL-1β, and TNF-α in unstimulated CD14$^+$ monocytes (C), in vitro stimulated CD14$^+$ monocytes (D), unstimulated CD16$^+$ monocytes (E), and in vitro stimulated CD16$^+$ monocytes (F) at 12 months after acute infection. * indicates unadjusted $P < 0.05$, ** indicates unadjusted $P < 0.01$, and *** indicates unadjusted $P < 0.001$ by Wilcoxon rank sum test. (**G** to **N**) Boxplots demonstrating expression during acute infection and 3 and 12 months after infection in CD14$^+$ monocytes are shown for *EPSTI1* (G), *GBP2* (H), *HERC5* (I), *IFI44L* (J), *IFIT3* (K), *LAP3* (L), *UBE2D1* (M), and *PARP9* (N). 3 mo, 3 months; 12 mo, 12 months. (**O**) *CD86* expression in CD14$^+$ monocytes is shown for samples collected at 3 and 12 months after acute infection. * indicates locally adjusted $P < 0.05$ for differential gene expression by MAST.

### Increases in NF-κB signaling are associated with LC

In addition to the pro-inflammatory role of NF-κB in monocytes from those with LC (Fig. 5B), sex-specific gene network analyses of a variety of cell types, such as $CD16^+$ monocytes and naive B cells in males at 3 months, and $CD8^+$ $T_{EM}$ cells in females at 12 months, revealed that *NFKB1* was up-regulated in those with ongoing LC (fig. S10). Given this, we next analyzed transcriptional expression of NF-κB transcription factors across cell types and time points. Given that we found that up-regulation of NF-κB constituents did not vary substantially by sex (fig. S11), the following analyses show the sexes combined for improved statistical power.

*NFKB1*, which is a primary component of the canonical NF-κB signaling pathway and induces cell proliferation and immune response programs (*81*), was found to have higher expression during acute infection in those who will develop LC at 3 months, as well as higher expression at 3 and 12 months in those with ongoing LC (Fig. 7A). Significant increases in *NFKB1* expression were observed at 3 and 12 months after infection in $CD8^+$ $T_{EM}$ cells (Fig. 7B) and during acute infection and 3 months in $CD4^+$ $T_{CM}$ cells (adjusted $P < 0.05$) (Fig. 7C). Consistently, *RELB*, an essential activator of transcription in the noncanonical NF-κB pathway (*82*), was up-regulated in most cell types at all time points (Fig. 7D). Last, *REL*, which is primarily expressed in B and T lymphocytes and has been implicated in multiple autoimmune conditions (*83*), also had higher expression in the acute samples of those who will develop LC and at 3 and 12 months in those with ongoing LC (Fig. 7E). Overall, widespread increases in several NF-κB transcription factor genes at multiple time points suggest an association between this pathway and LC pathogenesis.

### Enriched AP-1 and $T_H2$ immunity with reduced ETS1 in individuals with LC

BTM analysis at 3 months after acute infection in individuals who had ongoing LC at 12 months demonstrated increased expression of genes in the "signaling in T cells (I)" module (Fig. 5A). Inspection of this module revealed that four genes were implicated in a majority of these transcriptional differences: *FOS*, *FOSB*, *JUN*, and *JUNB* (fig. S12). These genes are major components of the activator protein-1 (AP-1) transcription factor complex, which plays important roles in T cell activation, and transcriptional expression was particularly increased at 3 months after infection in those with ongoing LC across many cell types (fig. S12) (*84*). Several of these genes also demonstrated increased expression in those with LC in gene network analyses of various cell types (fig. S10).

Given these findings corresponding to altered T cell function in LC and known physical and functional associations between AP-1 and the *ETS1* transcription factor (*85*), we evaluated pseudobulk *ETS1* expression in lymphocyte subsets in which it is known to be expressed (*86*). Comparison of *ETS1* in those with LC versus those who recovered revealed lower expression at 3 months in those who would have ongoing LC at 12 months, and it remained lower at 12 months in these individuals across most B and T cell subsets in both sexes (Fig. 8A).

Given that *ETS1* deficiency has previously been linked to increased T helper 2 ($T_H2$) cell differentiation (*87*), we assessed proteomic correlates to the *ETS1* reduction seen in our transcriptomic data. CyTOF data revealed that intracellular IL-4, a major $T_H2$ cytokine, was higher in $CD4^+$ and $CD8^+$ T cells at 3 and 12 months for individuals with ongoing LC at 12 months in both unstimulated and in vitro stimulated samples (Fig. 8, B to E). Plasma proteomics also revealed higher IL-33 abundance ($P < 0.05$), which is known to induce $T_H2$ cell signaling (*88*), in individuals with ongoing LC at 12 months (Fig. 8F). No difference was observed in IL-4, IL-5, or IL-13 abundance (Fig. 8F).

### $CD8^+$ $T_{EM}$ cells in LC expressed genes associated with ongoing antiviral responses

$CD8^+$ $T_{EM}$ cells had multiple genes with significantly increased expression [adjusted $P < 0.05$ by model-based analysis of single-cell transcriptomics (MAST) differential expression analysis] during acute infection and at 3 and 12 months in individuals with ongoing LC (Fig. 8, G to J). This set of genes includes *killer cell lectin like receptor C2* (*NKG2C*, also known as *KLRC2*) (Fig. 8G), which is an activating receptor classically expressed on NK cells, but it has also been found to be expressed on $CD8^+$ T cells that demonstrate greater cytotoxicity in individuals infected with cytomegalovirus infection (*89*). $CD8^+$ $T_{EM}$ cells in those with LC were also found to have ongoing increased expression of ATP binding cassette subfamily B member 1 (*ABCB1*, also known as *MDR1*) up to 12 months after acute infection (Fig. 8H). *ABCB1* is expressed in both NK and $CD8^+$ memory T cells, and it enhances survival of effector T cells in the presence of viral infection (*90*). Additional up-regulated genes at all time points in $CD8^+$ $T_{EM}$ cells included *integrin subunit alpha M* (*ITGAM*) and *C-terminal binding protein 2* (*CTBP2*) (Fig. 8, I and J), both of which act to increase IFN gene expression (*91*, *92*). In summary, $CD8^+$ $T_{EM}$ cells in those with LC show increased expression of several genes involved in host antiviral responses at all time points evaluated in this study.

## DISCUSSION

This study used multiomic data to examine individuals during acute infection and 3 and 12 months after infection to discover abnormalities in innate and adaptive immunity of individuals with LC, some of which differ by sex. BTM, cell-cell communication, and gene network analyses uncovered cell-specific as well as more pervasive differences in those who developed LC, such as increased expression of genes involved in TGF-β signaling in males, pro-inflammatory monocytes in both sexes, and NF-κB activation in both sexes. It also revealed reduced *ETS1* in both sexes in numerous lymphocyte subsets in the months after acute infection. Some of these transcriptional differences were corroborated by corresponding changes in single cell cytokine production and by plasma cytokines. Sex-specific immunologic differences were prominent across many cell types, indicating that mechanisms of LC may differ between sexes, potentially informing the search for tailored therapeutics.

Several genes with high specificity for biological sex were found to have expression differences that support roles in disease pathogenesis. In males, the *DDX3Y* gene was most reduced in those with LC at both 3 and 12 months, and the differences became most prominent in innate immune cells, such as $CD56^{bright}$ NK cells, cDC2s, and monocytes, at 12 months. *DDX3* genes have been shown to promote virus-induced IFN-β expression (*93*); therefore, reduced *DDX3* expression may have implications for clearance of residual SARS-CoV-2, suppression of other chronic viral infections, or response to new viral pathogens. In females, *XIST* was noted to have higher expression during acute infection and at 3 and 12 months for those who developed LC. The RNA product of *XIST* does not encode

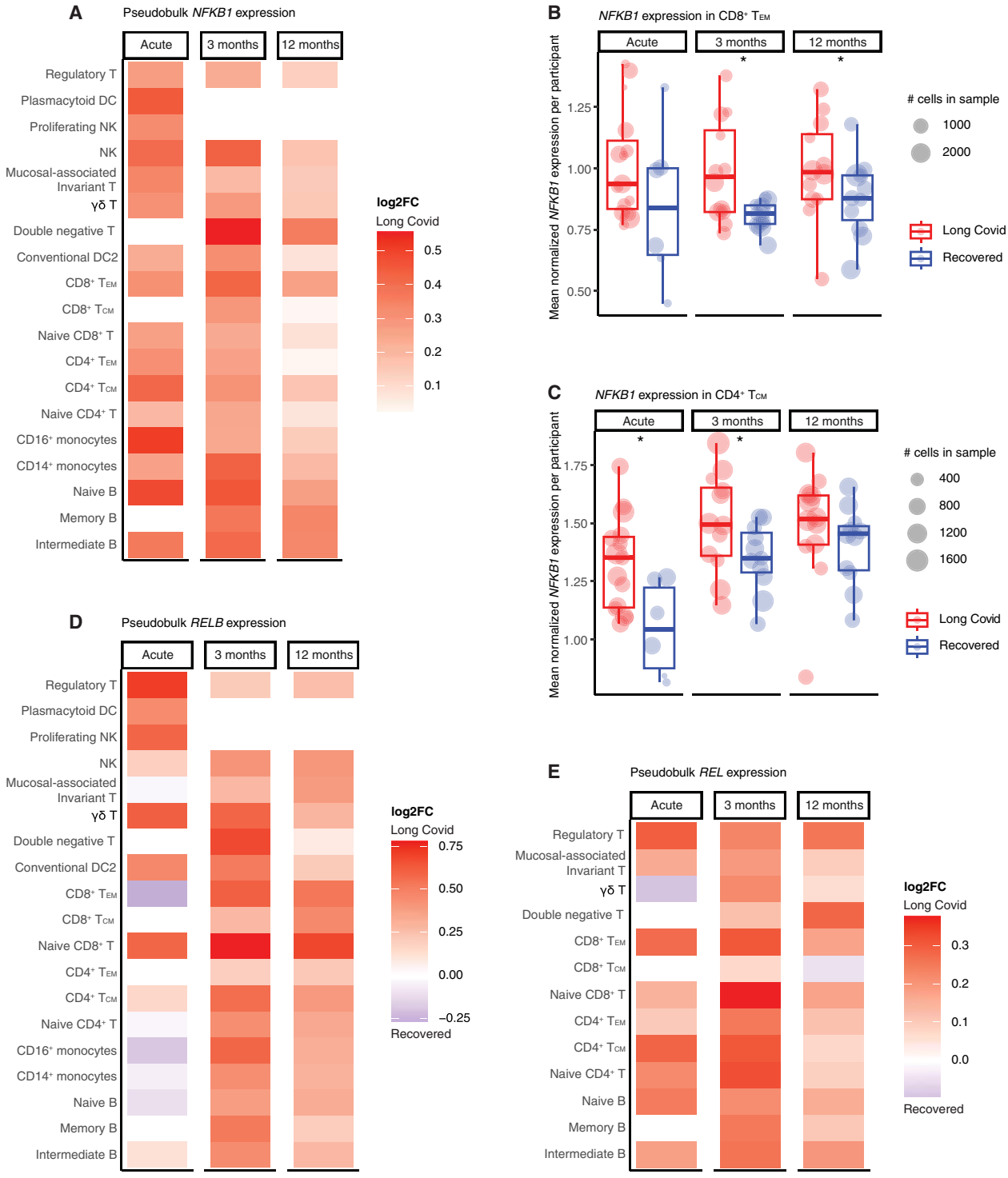



**Fig. 7. Increased expression of NF-κB transcription factors is associated with LC development and symptom persistence.** (**A**) Pseudobulk expression of *NFKB1* across cell types is shown in samples collected during acute infection ($n = 21$) and at 3 and 12 months after infection ($n = 23$). (**B** and **C**) Boxplots of *NFKB1* expression are shown by participants in $CD8^+$ $T_{EM}$ cells (B) and $CD4^+$ $T_{CM}$ cells (C) during acute infection and 3 and 12 months later. (**D** and **E**) Pseudobulk expression of *RELB* (D) and *REL* (E) is shown across cell types during acute infection and at 3 and 12 months after infection. For (A), (D), and (E), red tiles indicate higher expression (log2FC) in LC, and blue tiles indicate lower expression. * indicates locally adjusted $P < 0.05$ for differential gene expression by MAST.

a protein, but rather binds numerous other proteins and subsequently interacts with the X chromosome in females to silence gene transcription in one of the X chromosomes (*28*). This mechanism has been associated with markers of multiple autoimmune diseases, such as rheumatoid arthritis, SLE, and systemic sclerosis, and the Xist ribonucleoprotein complex has many sites of potential immunoreactivity

that may contribute to multiorgan autoimmune pathology (*28*). An association between autoantibodies and LC has been suggested in some studies (*4*, *10*, *94*), but not in others (*3*, *95*), necessitating further investigation that may benefit from considering sex-specific differences in titers, breadth, and association with LC symptoms.

In this cohort, there were perturbations in innate and early adaptive immunity during acute infection that correlated with development of LC. Proliferating NK cells in males showed increased expression of genes associated with bidirectional TGF-β signaling, which would be expected to reduce NK cell cytotoxicity, as previously observed in severe COVID-19 (*96*), and may limit the efficacy of the initial antiviral response. Differences in NK cell function between sexes have previously been demonstrated in the context of type I IFN responses and cytomegalovirus infection, showing that NK cells in females are

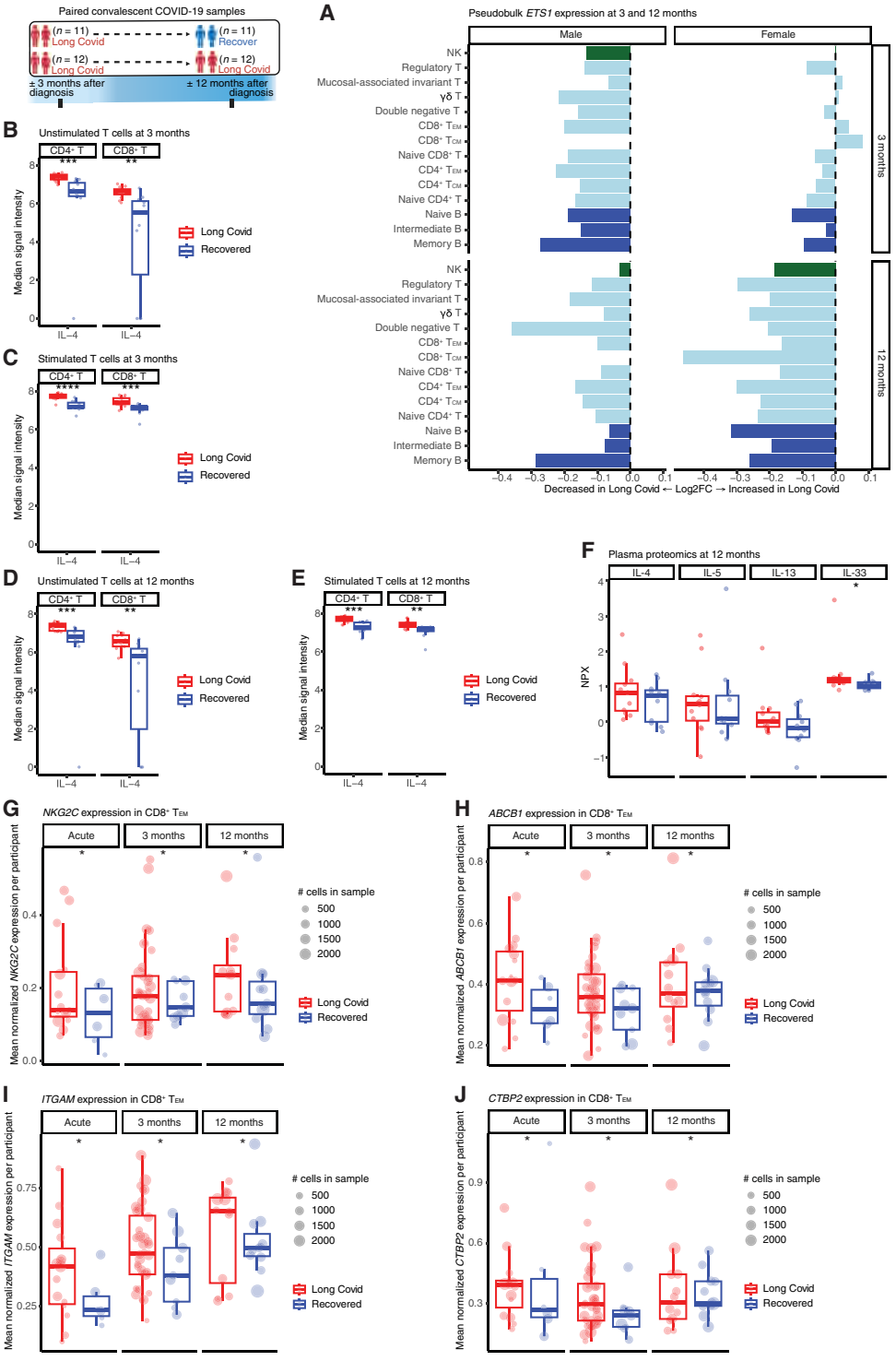



**Fig. 8. *ETS1* expression and IL-4 production distinguish those with persistent LC from those who recover.** (**A**) Log2FC of pseudobulk *ETS1* expression at 3 and 12 months after infection, comparing those with LC at 3 and 12 months versus those with LC at 3 months but recovery by 12 months, separated by sex. NK cells are represented by green bars, T cells by light blue bars, and B cells by dark blue bars. (**B** to **E**) Boxplots showing intracellular IL-4 as measured using CyTOF in $CD4^+$ and $CD8^+$ T cells at 3 months in unstimulated (B) and in vitro stimulated (C) samples and at 12 months after infection in unstimulated (D) and in vitro stimulated (E) samples, comparing those with ongoing LC at 3 and 12 months versus those with LC at 3 months but recovery by 12 months. (**F**) Boxplot of normalized protein expression (NPX) of common $T_H2$ cell immune cytokines IL-13, IL-33, IL-4, and IL-5 in plasma. * indicates unadjusted $P < 0.05$, ** indicates unadjusted $P < 0.01$, *** indicates unadjusted $P < 0.001$, and **** indicates unadjusted $P < 0.0001$ by Wilcoxon rank sum test. (**G** to **J**) Boxplots demonstrating $CD8^+$ $T_{EM}$ cell expression of *NKG2C* (G), *ABCB1* (H), *ITGAM* (I), and *CTBP2* (J) are shown by participants during acute infection and 3 and 12 months later. * indicates locally adjusted $P < 0.05$ for differential gene expression by MAST.

more responsive and cytotoxic in these settings (*97*, *98*). Here, increased *TGFB1* expression in males was also found to be associated with LC development across many cell types, including naive and memory T cells, which may lead to reduced $T_H1$ differentiation (*99*) and impaired immunity against intracellular pathogens. Conversely, TGF-β signaling contributes to regulatory T cell differentiation, which may be important for avoiding self-reactivity (*100*). TGF-β has been previously suggested to play a role in myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) (*101*) and was described to be increased in the plasma of males with LC in a study of a different cohort of patients with LC (*102*).

In convalescence, increased inflammatory signaling involving monocytes persisted to 12 months in those with LC, characterized by up-regulation of major factors such as *IL1B*, *NFKB1*, *RELB*, and *REL* in transcriptomic data as well as IL-6 and CXCL10 in single-cell proteomic data. Despite up-regulation of multiple pro-inflammatory markers, monocytes appeared to have dysfunctional interaction with T cells, because transcriptional analysis indicated reduced *CD86* costimulatory expression. Increased LAG3 was noted at 3 and 12 months in participants with ongoing LC. LAG3 is associated with suppressed and exhausted T cell phenotypes in the contexts of autoimmune disease, malignancy, and infectious disease, and it is under investigation as a target for cancer immunotherapies given its potential to reduce T cell cytotoxicity (*103*). However, a T cell exhaustion gene module was not differentially expressed in individuals with LC, suggesting that the increase in LAG3 protein may not indicate a typical T cell exhaustion phenotype.

Transcriptomic analysis of $CD14^+$ monocytes revealed higher expression of multiple ISGs during acute infection and 3 and 12 months later in individuals with LC. In conjunction with gene expression differences in $CD8^+$ $T_{EM}$ cells suggestive of ongoing antiviral responses in those with LC, these findings could be consistent with persistence of SARS-CoV-2 or reactivation of other viruses in those with LC (*11*). Therefore, the development and application of therapies aimed at improving cytotoxicity and viral clearance, potentially in enriched populations that show evidence of viral persistence or reactivation, may be of benefit in LC.

Single-cell transcriptomic analyses also revealed a broader pattern of reduced *ETS1* transcription factor expression in nearly all lymphocyte subsets to be associated with LC ongoing to 12 months. *ETS1* deficiency in humans has been characterized in the context of autoimmunity, where it was highly correlated with presence of autoimmune disease and disease severity (*87*). Furthermore, *ETS1* deficiency was strongly associated with increased $T_H2$ cell activity by biomarkers such as IL-4, IL-5, and IL-13 (*87*). *ETS1* deletion in mice results in high IL-4 signaling activity, $T_H2$ bias, and clinical features of autoimmunity (*87*). In our cohort, we found an increase in IL-4 protein expression across T cells in those with LC at 3 and 12 months, mirroring the reduced *ETS1* expression seen in these cells. Although IL-4 signaling is classically associated with $CD4^+$ T cell modulation, it also affects $CD8^+$ T cell function, where it induces prosurvival programs (*104*) but also reduces cytotoxicity in the setting of viral infection (*105*). Increased IL-4 expression in $CD4^+$ T cells has previously been associated with LC (*3*). IL-4 blockade has been shown to cause remission of autoimmune disease in *ETS1*-deficient mice, so it may offer a feasible intervention strategy in LC given the efficacy and safety of anti–IL-4 receptor therapy in humans (*106*).

This study has important limitations. First, the prevalence of LC in this clinical cohort was higher than in the general population. Although participants were recruited regardless of ongoing symptoms, selection bias among the participants may be present such that those with LC symptoms were more likely to volunteer to participate in the clinical cohort. Second, the participants enrolled in this study were first infected with SARS-CoV-2 early in the COVID-19 pandemic, before the widespread dissemination of COVID-19 vaccines. However, prevalence of LC has been relatively unchanged from 2022 to 2024 (*107*), suggesting that vaccination may not offer a magnitude of protection against LC development comparable to that against severe disease during acute infection and that ongoing evolution of SARS-CoV-2 may not have substantially affected post-acute sequelae. Although none of the study participants had received vaccination before the acute infection and 3-month blood sample collection, some of the study participants did receive interval vaccination before the 12-month blood sample collection. Additional studies of LC cohorts during subsequent SARS-CoV-2 variant epochs are needed to validate our findings. Next, one participant received an experimental therapy for acute COVID-19, favipiravir, before acquisition of the acute infection blood sample used for our analysis. Although this individual does not appear immunologically distinct from others in this study, it is possible that there were biological or clinical effects of this treatment. Other participants whose blood was profiled at 3 and 12 months after infection received acute COVID-19 treatments with peginterferon lambda-1a, favipiravir, or remdesivir. These acute infection treatments may have unknown long-term biologic effects, but these acute treatments did not correlate with appreciable effects on the immunologic findings in this study. An additional limitation that applies to all LC studies is the current lack of objective measures that can be used for diagnosis. Because we prioritized performing transcriptomic analyses of multiple time points and discovery of sex differences, this study was limited by small sample size. Circulating sex hormone measurements and menopausal status were not obtained in this study; however, both may be important mediators of sex-specific immune responses to SARS-CoV-2. Given that this study was conducted on a small single-center cohort, additional studies to validate these findings in more racially and ethnically diverse cohorts would be required before applying such findings to the clinical setting.

In summary, this study used multiple modalities to uncover patterns of immune dysregulation that correlate with LC development, symptom persistence, and resolution. Sex-specific differences in immune responses identified herein suggest that future studies should consider stratification by sex as a prespecified analysis, as supported by disproportionate impacts of severe acute disease in males (*18*) and LC in females (*19*). Last, these data offer several potential targets for therapeutic intervention for those suffering from LC.

## MATERIALS AND METHODS

### Study design

This study used samples collected from 45 participants enrolled in the prospective, observational cohort called IRIS. Adult participants were enrolled in this cohort approximately 3 months after symptomatic, polymerase chain reaction (PCR)–confirmed SARS-CoV-2 infection between August 2020 and December 2020 at Stanford University (*108*). Participants were recruited for the IRIS study in several ways: study outreach after participation in trials for acute COVID-19 therapies at Stanford University, physician referrals, and self-referrals after advertisement on a "Treat COVID-19" website and

outdoor tent. Protocols were approved by the Stanford Institutional Review Board (IRB 57036), and participants provided written informed consent. Exclusion criteria for the overall IRIS cohort were preestablished and included pregnancy or active drug or alcohol abuse. For this immunologic study, participants with immunocompromising conditions (e.g., malignancy, HIV, and solid organ transplant), on immunomodulatory medications (e.g., prednisone, mycophenolate mofetil, tacrolimus, and ibrutinib), or with medical conditions that may have accounted for their symptoms (e.g., cystic fibrosis and fibromyalgia) were also intentionally excluded to reduce confounding variables in the study. Participants were included in this study on the basis of the availability of longitudinal blood samples and the prioritization of those with the clearest clinical phenotypes (e.g., asymptomatic throughout the entire convalescent period assessed for the recovered participants). None of the participants were known to be reinfected with SARS-CoV-2 during the study period.

Study data were collected and managed using REDCap electronic data capture tools hosted at Stanford University (*109*, *110*). PBMCs and plasma were collected at approximately 3 months [median 99 days (range 57 to 184)] and 12 months [median 368 days (range 307 to 448)] after infection. Clinical assessments were performed using a combination of formal interviews, self-administered questionnaires, clinician-led physical exams, and complementary chart review. Evaluated clinical features included medical histories, physical exams, vital signs, and self-reported symptoms (both checklist survey and free text response) at approximately 3, 6, and 12 months after SARS-CoV-2 infection. Consistent with the World Health Organization (WHO) definition, LC was defined in this study as the continuation or development of symptoms 3 months after SARS-CoV-2 infection, which were not readily attributable to other etiologies (*111*).

Of the 45 participants in this study, 21 participants with banked PBMCs and plasma during acute SARS-CoV-2 infection were profiled in this work. Twelve of these participants had been previously enrolled in a phase 2, randomized, single-blind, placebo-controlled trial of single subcutaneous dose of peginterferon lambda-1a as a potential treatment for outpatients with mild to moderate COVID-19 (NCT04331899) at Stanford University (*112*). In addition, nine of these participants had been previously enrolled in a phase 2, randomized, double-blind, placebo-controlled trial of a 10-day course of oral favipiravir as a potential treatment for outpatient mild or asymptomatic COVID-19 (NCT04346628) at Stanford University (*113*). Neither study found a difference in the duration of SARS-CoV-2 viral shedding or in symptom duration between the treatment arm and the placebo arm (*112*, *113*).

### Statistical analysis

Individual-level data are presented in data file S1. Comparison of means was performed with the Wilcoxon rank sum test (two-sided), and symbols corresponding to $P$ value thresholds are noted in the figure legends (all <0.05). The threshold for statistical significance of differential gene expression for each cell type was determined as $P < 0.05$ with the Bonferroni correction. All data analysis was performed using R version 4.3.1 on a macOS when possible. For analyses that required additional memory or computer processing units, R version 4.2.0 was used on a Linux cluster.

## Supplementary Materials

**The PDF file includes:**
Materials and Methods
Figs. S1 to S15
Tables S1 to S7
References (*114*–*128*)

**Other Supplementary Material for this manuscript includes the following:**
Data file S1
MDAR Reproducibility Checklist

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**Acknowledgments:** We acknowledge all the participants in the IRIS cohort and in the peginterferon lambda-1a and favipiravir clinical trials. We are thankful to all the trial coordinators, laboratory personnel, and staff who contributed to the success of these trials and cohort. We thank the Stanford ChEM-H and Stanford Innovative Medicines Accelerator Clinical Research Coordinator. We are grateful to M. Davis and his laboratory for the use of their CyTOF machine. We thank M. Leipold for CyTOF training and expertise. We thank A. Rustagi for guidance with Parse Biosciences scRNA-seq library preparation and data preprocessing. We appreciate the assistance of N. Neff and A. Seng of the Chan Zuckerberg Biohub with sequencing. We also thank K. Ratnasiri for helpful discussions regarding scRNA-seq analysis and guidance in implementing BTM analysis. Figure schematics were created with BioRender.com. **Funding:** This work was supported by the Stanford Innovative Medicines Accelerator IMA-1035 (to C.A.B., A.S., P.G., H.B., and S.P.H.); National Institutes of Health grants OT2HL161847 (to U.S. and C.A.B.), T32AI007502 (to R.E.H.), 5T32HL129970 (to S.M.P.), 5T32AI007290-37 (to L.C.), and U01 AI150741-01S1 (to P.J.); and a Chan Zuckerberg Biohub Investigator Award (to C.A.B.). Anonymous donors to Stanford University provided funding support for the favipiravir and peginterferon lambda-1a clinical trials. Peginterferon lambda-1a was provided by Eiger BioPharmaceuticals. This work was also supported by the National Institutes of Health, National Center for Research Resources, and the Stanford Clinical and Translational Science Award UL1TR001085 (REDCap platform services at Stanford). **Author contributions:** R.E.H., S.M.P., S.P.H., A.S., P.G., H.B., and C.A.B. conceptualized the study. S.M.P., R.E.H., S.P.H., and C.A.B. developed the methodology. R.E.H., L.C., M.A.S., and K.P. performed experiments, generating the single-cell RNA sequencing and CyTOF data. S.M.P., R.E.H., S.P.H., and C.A.B. analyzed the data. P.G., A.S., H.B., M.H., U.S., K.B.J., P.J., Y.M., M.R., and C.A.B. provided resources. S.M.P., R.E.H., S.P.H., and C.A.B. visualized the data. C.A.B., A.S., P.G., H.B., U.S., and P.J. acquired funding. M.A.S., K.P., and M.R. performed project administration. A.S., C.A.B., and S.P.H. supervised the study. R.E.H., S.M.P., and C.A.B. wrote the original draft of the manuscript. S.M.P., R.E.H., L.C., M.A.S., K.P., M.R., P.G., A.S., H.B., M.H., U.S., K.B.J., P.J., Y.M., S.P.H., and C.A.B. reviewed and edited the manuscript. **Competing interests:** U.S. reports research support from the

National Institutes of Health, Agency for Healthcare Research and Quality, and Pfizer Inc.; she is an advisor to Regeneron and Gilead. C.A.B. is an advisor to Immunebridge and DeepCell on topics unrelated to this research. All other authors declare that they have no competing interests. **Data and materials availability:** All data associated with this study are present in the paper or the Supplementary Materials. Mass cytometry FCS files with deidentified metadata supporting this publication are deposited in ImmPort (accession SDY2757). Data from scRNA-seq are available with the Gene Expression Omnibus (accession GSE270479). Olink data with deidentified metadata are available at Zenodo (DOI: 10.5281/zenodo.13363117). All original code for analysis and visualization is available at Zenodo (DOI: 10.5281/zenodo.13733726). There were no proprietary materials used for data generation or analysis.

Submitted 14 June 2024
Accepted 18 October 2024
Published 13 November 2024
10.1126/scitranslmed.adr1032