Prospective studies suggest that one in eight people infected with the severe acute respiratory syndrome coronavirus type-2 (SARS-CoV-2) have persistent symptoms. This novel disease, known as long-COVID or post-acute COVID-19 syndrome (PACS), is more prevalent among hospitalization survivors. Still, even people who have experienced mild acute COVID-19 have a wide range of organ dysfunction and clinical symptoms. The underlying pathogenesis of post-COVID syndrome remains unclear. Current hypotheses discuss the persistence of virus or viral remnants in various tissues, the development or aggravation of autoimmunity, micro-clotting and platelet hyperactivation, immune dysregulation, the reactivation of non-SARS-CoV-2 latent viral infections, and widespread organ damage resulting from chronic inflammation. The aim of this study by authors from the United States was to identify biological (immunological, virological, or hormonal) features of long COVID syndrome diagnosed more than one year after acute infection.

SARS-CoV-2 is an enveloped, positive-sense, single-stranded RNA virus. Its genome encodes four structural proteins, namely the spike (S), envelope (E), nucleocapsid (N), and membrane (M) proteins. The S protein is a glycosylated homotrimer with each monomer composed of subunits S1 and S2, separated by host cell proteases.

About the study

The study enrolled 268 participants, categorized into five different groups: healthcare workers infected with SARS-CoV-2 before vaccination, patients diagnosed with long COVID syndrome, post-COVID patients from an independent study, healthy uninfected vaccinated controls, and vaccinated convalescent controls without post-COVID symptoms.

Vaccinated convalescent controls and post-COVID patients from an independent study had mild acute SARS-CoV-2 infections that did not require hospitalization. Most (76%) patients diagnosed with long COVID syndrome were infected in 2020 when the majority of cases were caused by the parental SARS-CoV-2 strain (WA-1).

On average, samples were collected more than a year after the acute COVID-19. Antiviral reactivity was investigated using three complementary methodologies, namely rapid extracellular antigen profiling (REAP), serum epitope repertoire analysis (SERA), and enzyme-linked immunosorbent assay (ELISA).

Results

The most common symptoms in the long COVID group were fatigue (87%), brain fog (78%), memory impairment (62%), and confusion (55%). The postural orthostatic tachycardia syndrome (POTS) was also frequently reported.

The analysis of the circulating immune cells

Some populations of circulating immune cells were significantly changed in patients with long COVID syndrome compared to vaccinated convalescent controls without long COVID symptoms. Long COVID patients had an increased number of non-conventional monocytes (CD14lowCD16high) and a decreased number of conventional type 1 dendritic (cDC1) cells, which are responsible for antigen presentation and cytotoxic T-cell priming. The numbers of neutrophils, eosinophils, conventional and intermediate monocytes, plasmacytoid dendritic, and cDC2 cells were not significantly different between the two groups.

The naïve CD4+ and CD8+ T-cells and naïve B-cells did not differ significantly between these groups. The analysis of the circulating B lymphocyte populations showed that long COVID patients had a higher median relative percentage of B lymphocytes in activated populations (CD86highHLA-DRhigh) and double-negative subsets (IgD–CD27–CD24–CD38–), as well as the absolute count of double-negative B-cells, than vaccinated convalescent controls. The circulating levels of other B-cell subsets did not differ between the two groups.

The analysis of the circulating T lymphocyte populations showed that long COVID patients had a reduced number of CD4+ T central memory cells (CD45RA–CD127+CCR7–) and an increased absolute number of exhausted CD4+ T-cells (PD-1+TIM3+) compared to

vaccinated convalescent controls. In addition, an increased number of $TIGIT^{+}CD8+$ T-cells was detected in the cerebrospinal fluid of long COVID patients, consistent with possible immune exhaustion.

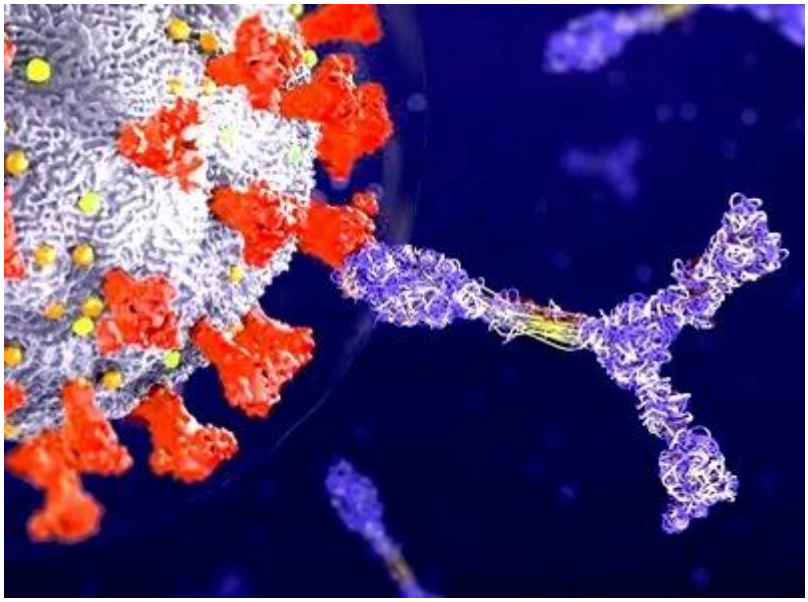
After stimulation with phorbol myristate acetate and ionomycin, CD4+ T-cells from individuals with long COVID syndrome produced higher median levels of intracellular interleukin (IL)-2 and IL-4. CD8+ T-cells produced higher median levels of IL-2 and IL-6 compared to vaccinated convalescents and unvaccinated healthy controls.

The levels of interferon (IFN)-γ and IL-17 (in CD4+ T-cells), as well as of tumor necrosis factor and granzyme B (in CD8+ T-cells), did not differ across groups.

The antibodies to SARS-CoV-2-specific antigens

An initial analysis of antibody responses to SARS-CoV-2-specific antigens was performed only in long COVID patients who received two COVID vaccines. Long COVID patients had higher IgG levels against the SARS-CoV-2 S1 protein than vaccinated convalescent controls. There was no difference in total levels of anti-S protein IgG and anti-RBD IgG between the two groups.

Importantly, anti-SARS-CoV-2 N protein IgG levels were higher in unvaccinated long COVID patients than in unvaccinated convalescent controls.



The antibodies to other viruses

The antibody responses to other viruses were analyzed only in study participants vaccinated with two doses of the COVID vaccine. The results showed reactivities against 38 viral epitopes in 98 long COVID patients and 38 controls. Long COVID patients had higher REAP scores for several herpes virus antigens, including the Epstein–Barr virus (EBV) minor viral capsid antigen gp23, the EBV fusion-receptor component gp42, and the varicella-zoster virus (VZV) glycoprotein E.

Importantly, the number of polyfunctional CD4+ T-cells co-expressing IL-4/IL-6, detected in long COVID patients, correlated with reactivity to EBV lytic antigens, but not with reactivity to SARS-CoV-2-specific antigens. According to the authors, there is a possible shift in CD4+ T-cell activation in response to EBV, as previously speculated in myalgic encephalomyelitis/chronic fatigue syndrome.

Since anti-EBV IgM levels were not elevated, and there was no evidence of EBV viremia in long COVID patients, the authors stated that the observed higher reactivity to lytic EBV antigens may be due to recent EBV reactivation, rather than acute infection.

To summarize, in patients diagnosed with long COVID syndrome, the results revealed an increased IgG reactivity to EBV and VZV surface antigens, without evidence of primary EBV infection or acute viremia.

The analysis of hormones and soluble immune mediators

Higher median levels of complement C4b, chemokines CCL4, CCL19, CCL20, galectin-1, a proliferation-inducing ligand (APRIL), and luteinizing hormone (LH), as well as lower median levels of IL-5, were found in patients diagnosed with long COVID syndrome. The role of a cytokine APRIL, a member of the tumor necrosis factor family, after SARS-CoV-2 infection is still not fully investigated. It is well-known that APRIL is expressed after viral

infections and plays an important role in B-cell response and antibody production.

Importantly, the circulating cortisol levels decreased markedly in both long COVID groups. This result remained significant even after adjustment for demographic and sample-collection time variations. There was no correlation between a decrease in cortisol levels and a compensatory increase in adrenocorticotropic hormone (ACTH) levels, suggesting an inappropriately blunted hypothalamic-pituitary axis response. The authors pointed out that ACTH has an extremely short half-life in plasma, potentially compromising these results.

Subsequent statistical modeling demonstrated an association between reduced cortisol levels and long COVID syndrome, taking into account differences in age, sex, body mass index, time of sample collection, and cohort. In this model, serum cortisol levels were the most significant predictor of long COVID.

Conclusion

This study identified immunological, virological, and hormonal features in patients diagnosed with long COVID syndrome more than one year after acute infection. Several mechanisms, such as the persistence of SARS-CoV-2 antigens, reactivation of latent herpesviruses, chronic inflammation, and decreased cortisol levels, may contribute to the pathophysiological mechanisms of this syndrome. The authors emphasized that the finding of persistently lower cortisol levels in patients with long COVID syndrome more than a year after the acute infection warranted further investigation.

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