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Opinion

Emerging roles of SARS-CoV-2 Spike-ACE2 in immune evasion and pathogenesis

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The COVID-19 pandemic, caused by SARS-CoV-2, has caused an estimated 5 billion infections and 20 million deaths by respiratory failure. In addition to the respiratory disease, SARS-CoV-2 infection has been associated with many extrapulmonary complications not easily explainable by the respiratory infection. A recent study showed that the SARS-CoV-2 spike protein, which mediates cell entry by binding to the angiotensin-converting enzyme 2 (ACE2) receptor, signals through ACE2 to change host cell behavior. In CD8$^+$ T cells, spike-dependent ACE2-mediated signaling suppresses immunological synapse (IS) formation and impairs their killing ability, leading to immune escape of virus-infected cells. In this opinion article, we discuss the consequences of ACE2 signaling on the immune response and propose that it contributes to the extrapulmonary manifestations of COVID-19.

Viral strategies of immune evasion

Infectious agents and cancer cells use multiple redundant mechanisms to escape the surveillance of the immune system. For instance, *Staphylococcus aureus* uses protein A to bind the **Fc fragment** (see Glossary) of antibodies and produces **superantigens** that disrupt T cell activation [1]. Another bacterium, *Neisseria meningitidis*, produces a protein that recruits the host protein factor H to avoid complement-mediated cell killing [2]. Cancer cells can use CD4$^+$CD25$^+$FoxP3$^+$ regulatory T cells to generate an immunosuppressive tumor microenvironment [3]. Viruses often escape immunity by directly infecting lymphocytes or other immune cells [4]. Of these, the infection and depletion of CD4$^+$ T cells by HIV-1 is probably the best example [5]. Viruses also produce a great variety of immunosuppressive factors. For instance, herpesviruses produce Fc-binding proteins which, similarly to the bacterial protein A, disarm antibodies [6]. Hepatitis C virus (HCV) uses the core protein to inhibit the maturation of cytotoxic CD8$^+$ T cells [7]. The infection and evolutionary success of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) suggests that the virus has found ways to circumvent immune defenses (Box 1). Indeed, the virus has been described to suppress monocyte interferon (IFN) production and signaling by impairing IFN regulatory factor 3 (IRF3) nuclear translocation using the nonstructural proteins Nsp6 and Nsp13; to inhibit IRF3 and STAT1 nuclear trafficking using the accessory protein encoded by open reading frame (ORF) 6, and to block **retinoic acid-inducible gene I/mitochondrial antiviral signaling (RIG-I/MAVS)** using ORF9b [8]. Furthermore, the spike protein of the virus, by binding to its receptor ACE2, was recently shown to inhibit the formation of the **IS** of human CD8$^+$ T cells with B cells (discussed later) [9]. In this opinion article, we focus on the possible consequences of spike signaling on ACE2-bearing cells upon receptor engagement; indeed, these cells are widely present in the human body and are susceptible to SARS-CoV-2 infection [10]. Moreover, SARS-CoV-2 infection has been linked to many extrapulmonary complications such as thrombotic complications, myocardial dysfunction and arrhythmia, acute coronary syndromes, acute kidney injury, gastrointestinal symptoms, hepatocellular injury, hyperglycemia

Highlights

CD8$^+$ cytotoxic T lymphocytes (CTLs) are major targets of SARS-CoV-2. Immunological synapse (IS) formation has emerged as an Achille's heel in COVID-19. Several accessory proteins disrupt antigen presentation by virally infected cells, and spike directly suppresses IS assembly by interacting with angiotensin-converting enzyme 2 receptor (ACE2) on CTLs.

While ACE2 was previously considered a component of the renin–angiotensin system, recent reports highlight a role for ACE2 as a signaling receptor that acts independently of its ectoenzyme activity.

Accumulating evidence shows a widespread distribution of ACE2 in different tissues and organs which are affected in COVID-19 patients, presumably contributing in part to extrapulmonary disease manifestations.

SARS-CoV-2 exploits both the structural and accessory proteins encoded by its genome to systematically subvert the innate and adaptive immune defenses of the host.

Significance

The recent finding that the SARS-CoV-2 spike protein exploits ACE2 signaling to suppress immunological synapse assembly and CD8$^+$ cytotoxic T lymphocyte-mediated killing highlights a potential role for the spike–ACE2 axis in the extrapulmonary manifestations of COVID-19.

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Key figure

Extrapulmonary disease manifestations and angiotensin-converting enzyme 2 (ACE2) expression/SARS-CoV-2 persistence in the human organs of COVID-19 patients

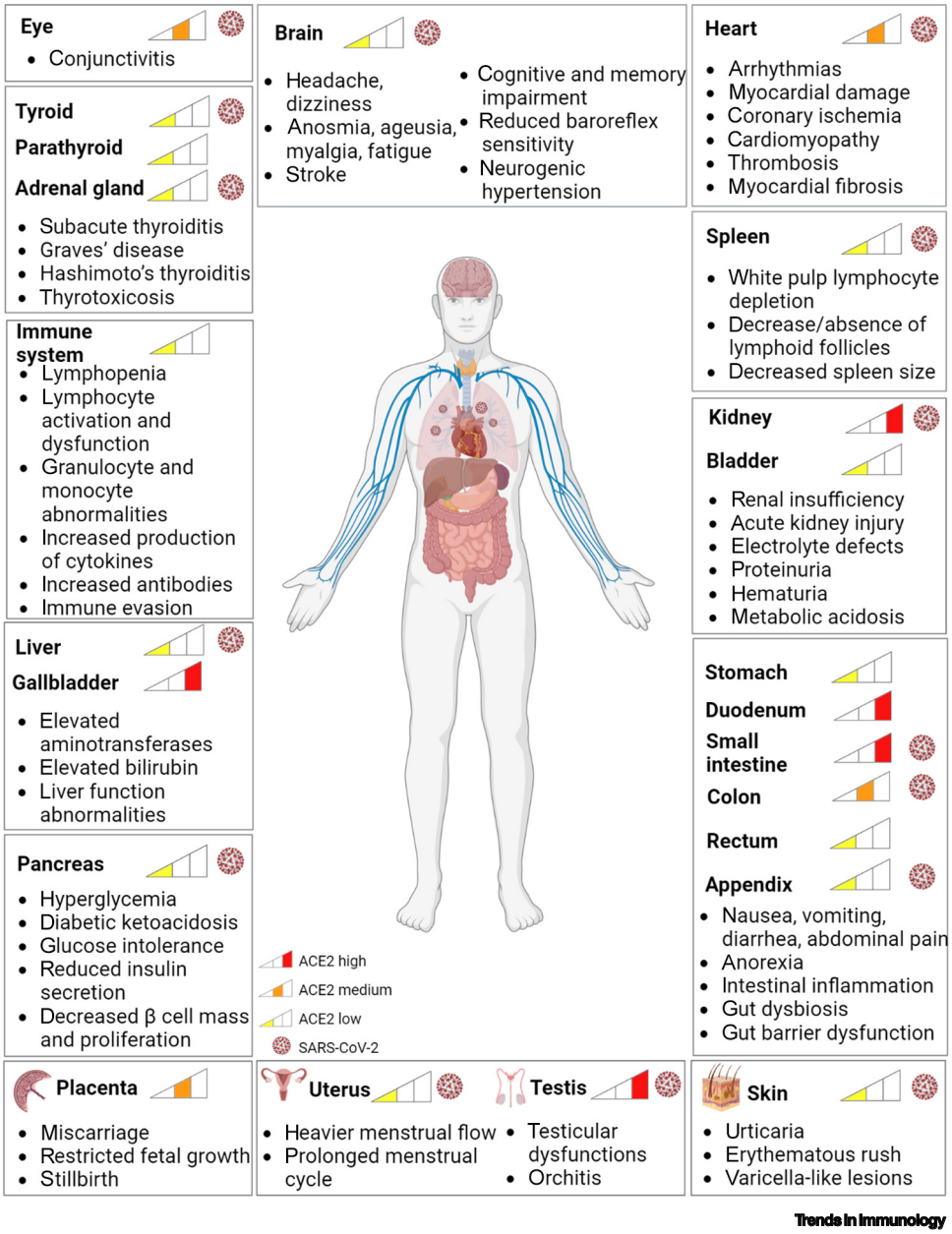

Figure 1. Schematic overview of the main extrapulmonary clinical manifestations of COVID-19. For each organ the amount of ACE2 protein expression is included from high to low using different colors (yellow = low, orange = medium, red = high). Documented SARS-CoV-2 persistence in each organ is also indicated. This figure was created using BioRender.com.

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and ketosis, neurological illnesses, ocular symptoms, dermatological and menstruation complications, as well as **long COVID** (Figure 1, Key figure). In this opinion article, we summarize the data showing that the spike protein inhibits the killing function of **cytotoxic T cells (CTLs)**, we suggest that this may explain the observed reactivation of some latent viruses during the coronavirus disease 2019 (COVID-19) pandemic, and we discuss how the action of the spike protein on ACE2 signaling in different tissues may be linked to many clinical complications that are difficult to explain by the infection of the respiratory tract alone.

Box 1. The immunopathology of COVID-19

SARS-CoV-2 infection has been associated with abnormalities in both innate and adaptive immune responses (Figure I), leading to severe and fatal COVID-19. The immunopathology of severe COVID-19 is typified by impaired induction of interferons (IFNs), exuberant inflammatory responses, profound lymphopenia, delayed adaptive immune responses, and production of autoantibodies [64–67]. The unbalanced orchestration of the innate response is implicated in the 'cytokine storm', a hallmark of severe COVID-19 [68–71]. High activation of $CD14^+CD16^+$ monocytes is responsible for the excessive production of proinflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) [68,69]. High neutrophil number promotes excessive formation of neutrophil extracellular traps resulting in autoantibodies and cytokine production, and possible activation of prothrombotic pathways [12,66,72,73]. Furthermore, neutrophils promote $CD4^+$ T cell polarization toward IL-17-producing T helper 17, leading to monocyte/macrophage recruitment to the site of infection and stimulation of IL-1β and IL-6 cytokine cascades [65,74–76]. In addition to the excessive proinflammatory responses, lymphopenia and exhaustion of natural killer (NK) cells (characterized by high expression of NKG2A and low production of granzyme B) are also distinctive traits of severe COVID-19 [14,77]. The dysfunctional immune response is also prominent in the adaptive immunity. Key features of severe COVID-19 are profound lymphopenia in severe infection (characterized by marked reduction in $CD4^+$ and $CD8^+$ T cells, B cells), T cell hyperactivation and exhaustion, and limited B cell maturation [9,64,65,78–81]. The inflammatory cytokine storm is likely an important factor behind the observed lymphopenia. In fact, high concentrations of IL-6 are associated with massive lymphocyte death [82]. Furthermore, the impaired production of type I IFN as a consequence of autoantibody production and decline of plasmacytoid dendritic cells blocks the expression of B cell lymphoma 6 (Bcl-6) in $CD4^+$ T cells, preventing T follicular helper (Tfh) differentiation and the development of a mature humoral response [80,83]. This scenario might help explain the lack of B cell maturation and the production of a germline-like antibody response observed following SARS-CoV-2 infection [82–84]. Another intrinsic aspect of COVID-19 is the development of autoantibodies detected in 5–10% of patients with severe or life-threatening COVID-19 pneumonia. These have been found to target mainly cytokines and chemokines involved in lymphocyte function and activation, and in leukocyte trafficking [67,84]. Taken together, the profound impairment of the immune response, compounded with the broad tropism of SARS-CoV-2 discussed herein, might be in part responsible for the multiorgan dysfunction observed in COVID-19 patients.

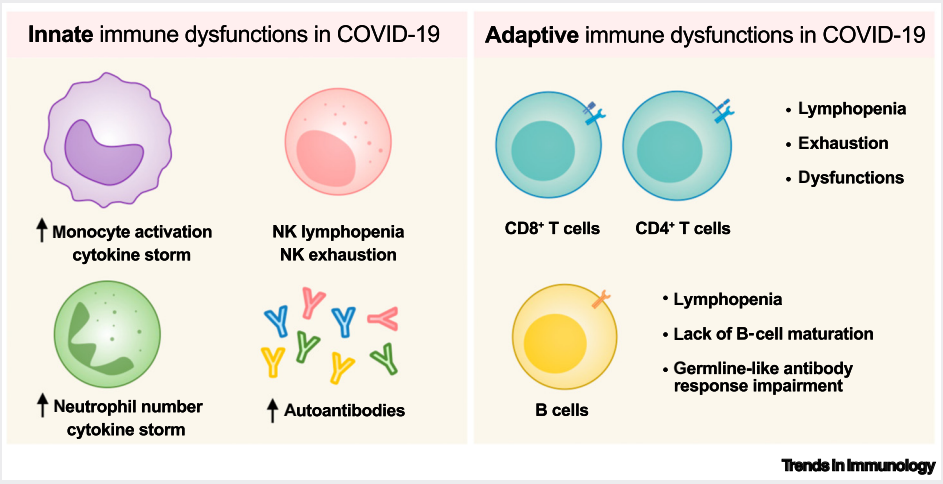

Figure I. Immune dysfunctions associated with COVID-19.

Glossary

Bacillus Calmette–Guérin (BCG): a live attenuated vaccine form of *Mycobacterium bovis* used to prevent tuberculosis disease.
Bronchoalveaolar lavage (BAL): a diagnostic medical procedure in which sterile normal saline is instilled into the lung with a flexible bronchoscope, followed by collection of the fluid enriched in immune cells which line the bronchial and alveolar spaces (macrophages, lymphocytes, and neutrophils).
Cytotoxic T cells (CTLs): T cells involved in adaptive immune responses and specialized in the killing of infected or cancerous cells.
Endocytosis: cellular process in which both extracellular material and plasma membrane proteins are internalized to form a vesicle and traffic inside the cell.
Exocytosis: cellular process in which secretory vesicles fuse with the plasma membrane and release their contents into the extracellular space.
Fc fragment: the fragment crystallizable region (Fc) is a constant region which determines the class of the antibody and its functional properties.
Histiocytic inflammation: disorder characterized by the pathological accumulation in normal tissue of cells derived from the monocyte and macrophage lineage, resulting in tissue damage.
Immune evasion: strategy adopted by pathogens and tumoral cells to evade the host immune response and ensure survival within the host.
Immunological synapse (IS): specialized signaling and secretory platform forming at the interface of a T cell with an antigen-presenting cell (APC) displaying cognate peptide antigen bound to MHC (pMHC). The mature IS features a bullseye architecture with a central supramolecular activation cluster (cSMAC) enriched in TCRs and costimulatory molecules such as CD28, a peripheral SMAC (pSMAC) enriched in integrins such as LFA-1, and a distal SMAC (dSMAC) enriched in actin filaments where surface molecules recruited to the IS assemble in microclusters; the latter are driven toward the IS with the help of the actin and microtubule cytoskeleton where bulky receptors are segregated to favor TCR–pMHC interactions at the cSMAC. During IS formation, the centrosome polarizes toward the APC, allowing for directional exocytosis.

Evasion of CTL-mediated antiviral defenses by SARS-CoV-2

$CD8^+$ CTLs, the adaptive immune cells responsible for viral clearance by killing infected cells, are strategic targets for immune escape by viruses. SARS-CoV-2 is no exception. Severe COVID-19 patients show selective reduction in the number of $CD8^+$ T cells in **bronchoalveaolar lavage (BAL)** fluid (BALF) and blood compared with patients with milder disease or healthy individuals [11]. Additionally, their cytotoxic function is impaired, as evidenced by the decreased proportion of $CD8^+$ T cells expressing the cytotoxic effector protein granzyme B and the degranulation marker CD107a [12–14]. Multiple direct and indirect mechanisms of CTL suppression, involving a variety of viral virulence factors, have emerged recently [15]. For example, ORF3a, ORF7a, and ORF8 have been reported to inhibit antigen presentation by the **major histocompatibility complex I (MHCI)** at different steps in human and primate kidney epithelial cell lines [15–17], leading to defective CTL activation. ORF3a appears to inhibit protein trafficking through the secretory pathway [16], ORF7b binds to MHCI to prevent its interaction with β_2 microglobulin which results in MHCI retention in the endoplasmic reticulum (ER) [17], and ORF8 promotes MHCI degradation via autophagy [18]. An interactome study also identified ORF3a as an interactor of ZNF579, a transcription factor whose targets are selectively enriched in $CD8^+$ T cells from SARS-CoV-2-positive versus -negative BALF derived from COVID-19 patients. ZNF579 is degraded when bound to ORF3a [19], suggesting that its depletion may affect the expression of genes relevant to $CD8^+$ T cell function in the infected lung (Figure 2). Thus, there is increasing interest in the role of SARS-CoV-2 accessory proteins in COVID-19 pathogenesis, and these proteins are revealing unexpected effects in key cellular processes, as suggested by interactome studies [19–22] and exemplified by the preliminary study on ORF3a-mediated inhibition of **vesicular trafficking** [16]. Such findings are likely to shed light on various immune dysfunctions associated with SARS-CoV-2 infection.

We recently identified an additional mechanism of SARS-CoV-2-mediated CTL suppression driven by the binding of the spike protein to ACE2 [23], which promotes SARS-CoV-2 uptake in airway epithelial cells [24,25]. ACE2, which we found was expressed on human $CD8^+$ T cells during their differentiation into CTLs [25], prevented the assembly of a functional IS when bound to the spike protein in an experimental setting; specifically, CTLs differentiated from primary peripheral-blood $CD8^+$ T cells were mixed with B cells loaded with a mix of staphylococcal superantigens (SAgs), used as antigen-presenting cells (APCs) [9]. The IS is a highly structured signaling and effector platform that forms at the T cell interface with APCs displaying specific peptide–MHC complexes (Figure 2). In CTLs, the IS acts as a focal point for **exocytosis** of their cytotoxic effectors, which are enclosed in specialized lysosomes known as lytic granules. The latter are well established to be enriched in perforin (pore-forming protein) and granzymes (proteolytic enzymes). The perforin-mediated entry of granzymes into target cells triggers an apoptotic cascade that results in their elimination [25]. Using soluble trimeric spike or synthetic particles carrying multiple copies of spike, we showed that spike suppressed IS assembly in CTLs by interfering with T cell receptor (TCR) recruitment and activation of the tyrosine phosphorylation cascade required for lytic granule positioning beneath the IS, and their local delivery [25,26]. These defects in CTL IS assembly resulted in their impaired ability to kill target cells, as evidenced from flow cytometry and fluorimetry-based killing assays using either CTLs generated from peripheral-blood $CD8^+$ T cells and SAg-loaded B cells, or an antigen-specific CTL line, along with matched target cells. In this study, **neutralizing antibodies** that prevent spike binding to ACE2 restored IS assembly, as assessed by imaging the TCR recruitment and signaling at the CTL contact with the target cell, as well as by examining lytic granule polarization to, and exocytosis at, the IS [25]. Although the molecular determinants of spike that are recognized by these antibodies may potentially bind other surface receptors, these findings nevertheless suggest that the suppressive effects of spike on CTLs are mediated by ACE2. In support of this notion, human quiescent CTLs, that do not express ACE2 [9], were not affected by spike, but recapitulated the spike-induced IS defects observed in

Long COVID: long-term effects of COVID-19 that persist for weeks or months beyond the initial illness.
Major histocompatibility complex I (MHCI): a heterodimeric transmembrane protein with antigen-presenting capabilities expressed in almost all nucleated cells and platelets. Intracellular antigens are fragmented by proteolysis into peptides which bind MHC-I to be presented on the cell surface and are recognized by the TCR of $CD8^+$ T cells to induce adaptive immunity.
Neutralizing antibodies: antibodies that bind either the cellular receptor exploited by a pathogen for entry into the host cell, or directly to the pathogen to protect the host from infection.
Retinoic acid-inducible gene I/mitochondrial antiviral signaling (RIG-I/MAVS) pathway: a signaling pathway triggered in target cells involved in sensing RNA virus infections, mediating the transcriptional induction of type I interferons and other genes to initiate and modulate antiviral immunity.
Shedding: a mechanism for removal of the extracellular domain of transmembrane proteins by proteolysis.
Superantigens: a class of antigens produced by pathogens capable of stimulating a large fraction of T cells in a TCR β-chain variable domain (Vβ)-dependent manner.
Supported lipid bilayers: a method to image the immunological synapse (IS) in the absence of an antigen-presenting cell using lipid bilayers embedded with free-moving ligands for the TCR, integrins, and other costimulatory molecules participating in IS formation, acting as an artificial IS. Imaging by total internal reflection fluorescence microscopy allows for a fine resolution of IS architecture.
Type 17 helper (Th17) cells: a specialized effector $CD4^+$ T cell lineage characterized as preferential producers of interleukin 17 and 17F. The effector cytokines mediate host defensive mechanisms to various infections, especially extracellular from bacteria, and play indispensable roles in tissue immunity.
Vesicular trafficking: a cellular process in which cargoes (proteins, pathogens, or macromolecules) move between subcellular compartments (e.g., endoplasmic reticulum, Golgi) using membrane vesicles that traffic to and from the plasma membrane to ensure correct cargo localization within the cell or its release to the extracellular milieu in a highly organized manner.

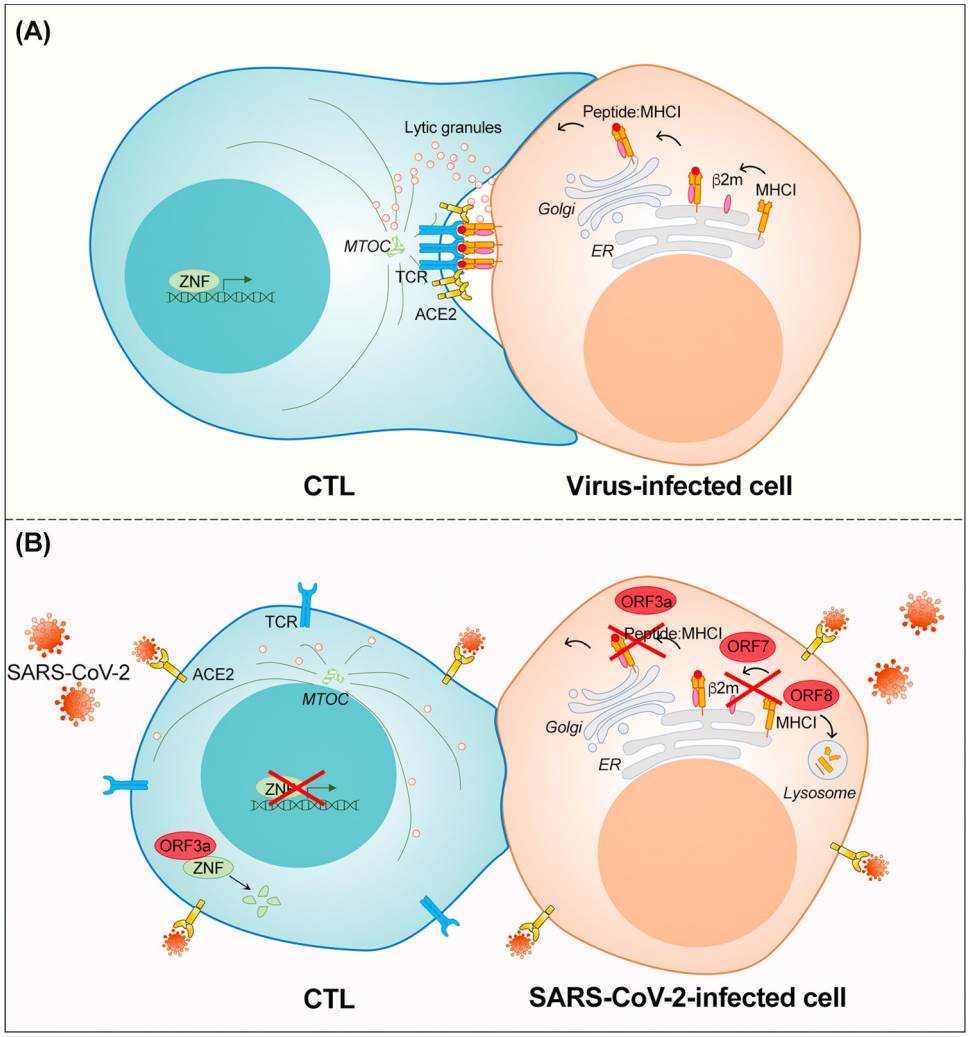

Figure 2. The immunological synapse (IS) that is formed by human cytotoxic T cells (CTLs) with virally infected cells is a direct and indirect target for SARS-CoV-2 immune evasion. (A) During viral infections, activated CTLs form a specialized interface with virally infected cells called the IS to promote their apoptotic demise. Formation of a mature IS is triggered by T cell receptor (TCR) engagement by viral major histocompatibility complex I (MHCI)-bound peptides on the target cell. This event leads to the IS clustering of TCR:CD3 complexes and accessory receptors, including ACE2, to coordinate CTL activation. Additionally, CTLs reorganize their cytoskeleton, and the microtubule-organizing center (MTOC) reorients toward the IS to ensure the polarized secretion of lytic granules into the synaptic cleft to selectively kill the infected target cell. (B) Defective IS formation caused by defective CTL signaling or impaired MHCI processing and presentation on target cells may lead to immune evasion and survival of infected cells. SARS-CoV-2 has adopted several strategies to bypass immune responses and persist in the host. In CTLs, SARS-CoV-2 open reading frame 3a (ORF3a) protein interacts with the CTL transcription factor ZNF579 to promote its proteolysis. Additionally, the SARS-CoV-2 spike protein binds ACE2 on activated CTLs, affecting IS assembly [9,25]. In target cells, SARS-CoV-2 accessory ORF proteins downregulate MHCI expression using different mechanisms: (i) ORF3a may induce degradation of the Golgi apparatus, impairing MHCI trafficking to the cell surface, (ii) ORF7a interacts with the MHCI heavy chain in the ER, inhibiting its association with β2m and thus peptide loading, and (iii) ORF8 interacts with MHCI molecules for selective targeting for lysosomal degradation via autophagy [15–18]. Abbreviations: ER, endoplasmic reticulum; ZNF, zinc finger protein.

CTLs following forced expression of ACE2 [9]. Of note, while ACE2 expression in activated T cells has been previously correlated to their susceptibility to SARS-CoV-2 entry [24,27], a recent report indicated that ACE2 is not required for human T cell infection, based on the ability of the virus to

infect a T cell line lacking ACE2 [28]. Thus, this raises the possibility that receptors other than ACE2 might also contribute to viral entry and T cell suppression, although this remains to be investigated.

Possible consequences of CTL suppression: reactivation of latent infections

Does this functional impairment of $CD8^+$ T cells upon SARS-CoV-2 infection translate into clinical consequences for COVID-19 patients? It is well known that $CD8^+$ T cells play a major role in the containment of virus replication during subclinical, chronic, life-long infections, and that one of the first clinical signs of CTL malfunction is the reactivation of latent viruses. For instance, the varicella zoster virus (VZV), after causing chickenpox mainly during childhood, remains silent for decades in the sensory ganglia of human peripheral nerves [29]. However, a decline in immunocompetence, caused by aging and/or immunosuppression (due to medical conditions or pharmacological interventions), can reactivate the virus, causing shingles (herpes zoster) [30]. Indeed, some reports have documented an increased incidence of shingles days or weeks after the diagnosis of SARS-CoV-2 infection. In these studies, most cases of herpes zoster symptoms were mild to moderate; however, more severe manifestations (e.g., herpes ophthalmicus with eye complications) were reported in fully immunocompetent subjects [31]. The mechanisms behind this increased incidence of shingles during COVID-19 infection have remained elusive until now. As mentioned, because SARS-CoV-2 can infect the ganglia of peripheral nerves [32], it is logical to hypothesize that the presence of the SARS-CoV-2 virus in these ganglia might negatively act on the functionality of VZV-specific $CD8^+$ T cells when engaging the spike protein, causing the reactivation of the resident VZV and the appearance of clinically overt herpes zoster symptoms and signs, such as skin lesions. However, it remains to be determined if and whether this functional impairment affects all $CD8^+$ T cells or whether it involves specific subpopulations (perhaps resident memory cells) that are directly involved in the containment of chronically silent VZV.

Like VZV, herpes simplex virus (HSV) can remain latent for a long period of time and reappear periodically, causing mucocutaneous manifestations or, in more severe cases, pneumonitis or encephalitis [33]. Several studies have reported the reactivation of HSV, mainly HSV-1, in COVID-19 patients [34,35]. In most cases, these manifestations have been limited to mucocutaneous lesions or to ocular keratitis [34,35]. However, severe HIV-1-associated pneumonitis was reported in severely ill COVID-19 patients who were intubated in the ICU [36,37]. In a study of invasively ventilated COVID-19 patients, pulmonary HSV-1 reactivation was observed in 15 out of 18 patients analyzed, 11–40 days post-COVID-19 diagnosis [38]. Moreover, a significant increase in activated $CD38^+HLADR^+CD8^+$ T cells was reported in peripheral blood relative to HSV-negative controls. In addition, the expression of IFN-stimulated genes was significantly lower following HSV-1 detection [38]. Collectively, these findings suggest that SARS-CoV-2-driven immunosuppression might contribute to the late reactivation of HSV-1 in COVID-19 patients, although this warrants further investigation. Thus, as in the case of VZV, the presence of the SARS-CoV-2 spike protein might have contributed to the reduced functional activity of HSV-1-specific $CD8^+$ T cells with final reactivation of the virus that was reported in COVID-19 patients [34,35]. We argue that, in the most severely ill COVID-19 patients in the ICU, this spike-driven immunosuppressive effect of $CD8^+$ T cells in the respiratory tract might have been amplified, potentially due to the immunosuppressive pharmacological treatment of individuals with corticosteroids and/or other agents, although this remains conjectural [35]; this in turn, may have led to HSV-1 reactivation and sequelae such as symptomatic clinical manifestations, including severe ones such as pneumonitis.

From another angle, certain bacterial infections have been reported to be reactivated by SARS-CoV-2 infection. For instance, in humanized ACE2 transgenic C57BL/6 mice previously infected with *Mycobacterium tuberculosis*, subsequent superinfection with SARS-CoV-2 induced an increased

bacterial load in *M. tuberculosis*-infected lung tissues and bacterial dissemination, concomitant with decreased **histiocytic inflammation** in the lungs, compared with uninfected controls [39]. In addition, SARS-CoV-2 infection increased **bacillus Calmette–Guérin (BCG)**-induced **type 17 helper (Th17) T cell** responses while decreasing production of type 1 and increasing production of type 2 cytokine – such as interleukin 10 (IL-10) and IL-13 – in *M. tuberculosis*-infected mice; this reduced the efficacy of the BCG vaccine. In a human study, *M. tuberculosis*–SARS-CoV-2-coinfected COVID-19/tuberculosis symptomatic patients exhibited a reduced frequency of *M. tuberculosis*-specific $CD4^+$ T cells in lungs relative to SARS-CoV-2-negative patients; hypothetically, this might contribute to tuberculosis progression in these patients, although this remains to be documented [40]. Extensive clinical studies on tuberculosis–SARS-CoV-2 coinfection have been carried out since the beginning of the COVID-19 pandemic [41–43]. However, although COVID-19 might contribute to the progression – and possibly worsening – of tuberculosis, other factors might also contribute, such as pharmacological treatments, socioeconomic conditions, or other coinfections [43]. Moreover, while the effects of the spike–ACE2 axis on $CD4^+$ T cells remain to be addressed, these cells use the same building principles as CTLs to assemble ISs. Therefore, it might not be surprising if the inhibitory effects of spike might extend to $CD4^+$ T cells, which may represent an area of fruitful investigation (see Outstanding questions).

ACE2 signaling functions

How does ACE2 contribute to CTL IS assembly?

The ACE2-dependent mechanism that underpins spike-mediated suppression of TCR signaling and subsequent impairment of the cascade of events leading to CTL-mediated target cell killing remains to be elucidated. One important clue is the finding that ACE2 accumulates at the CTL IS and that this event is prevented by spike [9]. This suggests that, in the absence of specific ligands, ACE2 is recruited to the IS to modulate signals that dictate IS formation, while this does not occur following ligand binding. Consistent with this hypothesis, we found that treatment of primary human CTLs with an antibody against the extracellular domain of ACE2 recapitulated the effects of spike on IS assembly and CTL-mediated killing [9].

Until the discovery that ACE2 mediated SARS-CoV-2 entry into airway epithelial cells through **endocytosis** [24], the main function of this receptor had been viewed in the context of the renin–angiotensin system, where ACE2 acts as an ectoenzyme that uses angiotensin II to catalyze the production of the bioactive peptide Ang 1–7 [44]. Of note, we found that angiotensin II, but not Ang 1–7, suppressed IS assembly in CTLs [9], which is interesting as it rules out enzymatic activity of ACE2 in this function, further supporting the notion that ACE2 engagement is responsible for the inhibitory effects of spike and other ACE2 ligands on the CTL IS. Hence, to understand how spike binding to ACE2 interferes with IS assembly, it is essential to elucidate the enzyme-independent functions of ACE2. One such function might be integrin activation, which is an important prerequisite for IS formation [45]. It has been shown that β1 integrin interacts with ACE2 purified from failing human heart or with ACE2 overexpressed in HEK293 cells through two distinct integrin-binding sites in the extracellular domain of ACE2 [46] (Figure 3). Additionally, surface ACE2 enhances cell adhesion and modulates integrin signaling [39]. Whether ACE2 can activate integrins other than β1, such as the β2 integrin LFA-1 that participates in IS assembly, remains to be established. Although we did not observe any significant effect of spike on the ability of CTLs to interact with target cells [9], integrin activation by ACE2 in CTLs is a possibility that deserves to be addressed. If this were the case, the IS defects elicited by spike in CTLs would need to be assessed in more depth using methodologies such as **supported lipid bilayers** and total internal reflection microscopy (or other high-resolution microscopy methods) coupled with biochemistry; this would allow better characterization of integrin activation and the resulting adhesive interactions established by CTLs with epithelial cells or APCs that might be relevant in lung immune responses.

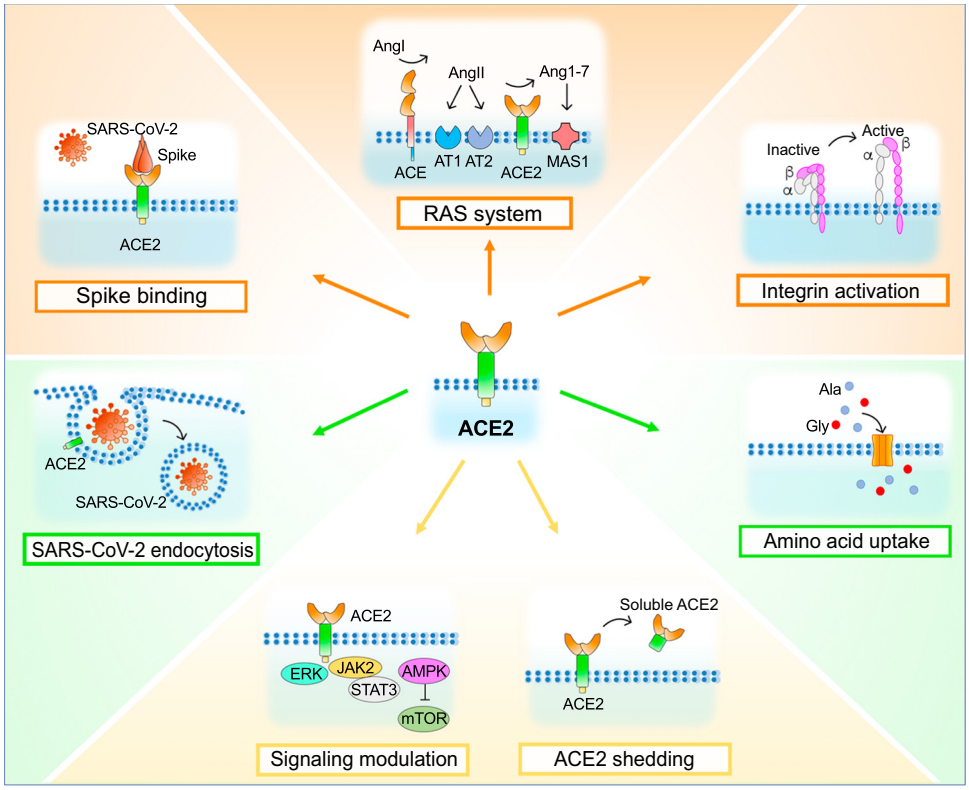

Figure 3. Human angiotensin-converting enzyme 2 (ACE2) domains and functions. ACE2 is a zinc metallopeptidase, a type-I transmembrane protein comprising a large extracellular domain (orange), a membrane-spanning collectrin homology domain (green), and a short cytoplasmatic tail (yellow) involved in several functions. The N-terminal extracellular domain has two lobes forming the peptide substrate binding site and is the only ACE2 domain harboring peptidase activity. The extracellular domain is also implicated in peptidase-independent functions such as spike binding and integrin activation. Additional peptidase-independent functions of ACE2 are mediated by the collectrin homology domain (SARS-CoV-2 endocytosis and amino acid transport) and the cytoplasmatic tail (signaling modulation and its own shedding) [47]. Colored arrows show the respective functions of the individual domains.

Regulation of amino acid homeostasis by ACE2

Peptidase-independent signaling functions of ACE2 have emerged with the finding that ACE2 resembles a chimeric protein in which the extracellular enzymatic domain is highly homologous to ACE, another key component of the renin–angiotensin system, while the transmembrane and intracellular domains display high homology with collectrin [47] (Figure 3). Collectrin is implicated in kidney and pancreas development and function; its main function in the kidney is to act as a chaperone to stabilize transporters of large neutral amino acids at the plasma membrane, thereby regulating amino acid transport [48,49]. Additionally, it participates in kidney homeostasis by coordinating primary cilium assembly [50]. Collectrin also contributes to the insulin secretory pathway by regulating its exocytosis through its association with snapin, a component of the SNARE complex [51]. The conserved nature of the entire collectrin amino acid sequence in mammalian ACE2 suggests that it might participate in processes regulated by collectrin, a concept that is supported by its involvement in amino acid uptake in the gut, which in turn, by using ACE2 knockout C57BL/6 mice, has been linked to the gut microbiota, intestinal homeostasis, and innate gut immunity [52]. This collectrin domain-related function of ACE2, which is abundantly expressed in the gastrointestinal tract, might contribute to intestinal manifestations of COVID-19.

ACE2 in intracellular signaling

A role for ACE2 as a canonical signaling receptor is slowly emerging. One documented function is the regulation of its own **shedding** by the metalloproteinase ADAM17 at the plasma membrane of ACE2-overexpressing human HEK293 cells [53]. This process is influenced by its interaction with calmodulin through ACE2's intracellular domain [53]. Additionally, the intracellular domain of ACE2 contains several putative phosphorylation sites that might couple the receptor to intracellular signaling modules. Inhibition of signaling pathways by ACE2 has been reported, including, among others, JAK2/STAT3 signaling in human umbilical artery vascular smooth muscle cells, AMPK/mTOR and ERK1 signaling in rodent models of lipopolysaccharide (LPS)-induced lung injury, and ERK signaling in human breast cancer cell lines [54–57], supporting a signaling function for ACE2 (Figure 3). However, whether these effects are directly mediated by ACE2 (acting as a signaling receptor) or indirectly (by Ang 1–7) remains to be thoroughly investigated. In addition to the respiratory tract, ACE2 is highly expressed by a variety of human tissues and organs, including the gastrointestinal, urogenital, circulatory, endocrine, and central nervous systems [58–61] (Figure 1). Of note, this has been recently matched to the SARS-CoV-2 tropism for multiple human non-respiratory tissues such as the brain, heart, small intestine, and adrenal glands, where it can be detected for several months [62] (Figure 1). Elucidating the role of each ACE2 domain in its increasing functional complexity is essential not only to delineate the mechanisms that are targeted to elicit immune dysfunction, but also to achieve insights into the extrapulmonary manifestations of COVID-19.

Concluding remarks

Since the sudden burst and rapid spread of the COVID-19 pandemic, overwhelming evidence has accumulated and been documented: on the one hand, a systematic subversion of the innate and adaptive immune response to SARS-CoV-2, and on the other its association with a wide variety of pathological manifestations occurring in organs that are not obvious targets of the virus, and which play a prominent role in disease severity, persistence, and sequelae. These intriguing findings have triggered the interest of the scientific community to elucidate the underpinning molecular mechanism, and which may help improve strategies for disease prevention and treatment. The past 2 years have witnessed a flurry of studies which have started to delineate the complex interactions of SARS-CoV-2 with its host, but which have also raised a number of major questions on how the virus exploits both its known proteins, such as spike and the nucleoprotein, and the proteins encoded by its as yet largely uncharacterized ORFs. Questions have also surfaced on how the virus evades immune defenses of the host and disrupts directly or indirectly the function of multiple organs beyond the respiratory system (see Outstanding questions). Our finding that the spike–ACE2 interaction is exploited by SARS-CoV-2 not only for cell entry but also to impair CTL-mediated killing by suppressing IS assembly has provided an important breakthrough in our understanding of the **immune evasion** strategies of the virus. While the study specifically dissected the role of spike in CTL suppression, other viral proteins may contribute to this function. Recent unbiased high-throughput studies have highlighted unexpected interactions of a variety of SARS-CoV-2 proteins with host proteins implicated in major cellular processes, from gene transcription to cell signaling, cytoskeleton dynamics, and vesicular trafficking [19,63]. Additionally, the striking widespread distribution of ACE2 and its emerging role as a signaling receptor beyond the renin–angiotensin system suggest that ACE2 ligands may affect not only CTL function but also key processes in cells expressing the receptor in a cell-context-specific fashion; these might account for the wide variety of extrapulmonary manifestations of COVID-19. A global understanding of how SARS-CoV-2 interfaces with its host is required to instruct both vaccine design and putative therapies.

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Outstanding questions

Does spike/ACE2 signaling affect CD4$^+$ T cells? To deliver help, effector CD4$^+$ T cells form immunological synapses (ISs) with their cellular targets. Given the key role of these cells in B cell maturation, ACE2 engagement by spike might have a major impact on the humoral response in COVID-19.

Does the state of spike/ACE2-dependent CTL suppression persist after recovery? While CTL suppression likely persists during active disease, how long this state may last after recovery is unknown. A 'memory' dictated by epigenetic reprogramming may have potential long-term consequences on the antiviral response in recovered COVID-19 patients. Of note, SARS-CoV-2 has been detected in target tissues several months after recovery.

What are the implications of ACE2 signaling and tissue distribution? ACE2 as well as SARS-CoV-2 have been detected in a variety of tissues, supporting a contributory role for ACE2 in COVID-19 extrapulmonary manifestations. Several points need to be addressed: (i) the effects elicited by ACE2 signaling must be discriminated from those related to its function in the renin–angiotensin system, (ii) since ACE2 is regulated not only transcriptionally but also epigenetically and post-translationally through shedding and degradation, studying ACE2 signaling requires determination of its surface expression, and (iii) whether biologically active concentrations of spike or other SARS-CoV-2 proteins can reach organs that are not viral targets should be considered. Determining whether the interaction of ACE2 with other coronaviruses leads to a similar array of pathological manifestations might help further elucidate the role of ACE2 in SARS-CoV-2.

What is the role of the viral accessory proteins in COVID-19? Accessory proteins of SARS-CoV-2 are emerging as important disease modulators. Understanding their role may provide new targets for therapeutic intervention. Information is missing on where these proteins are localized in the cell, which are their cellular interactors, and how these interactions modify cellular functions. Some of these factors are secreted (e.g., ORF8), also raising the question of which are their receptors on host cells.

Declaration of interests

No interests are declared.

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