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COVID-19 vaccines: The status and perspectives in delivery points of view

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ABSTRACT

Due to the high prevalence and long incubation periods often without symptoms, the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has infected millions of individuals globally, causing the coronavirus disease 2019 (COVID-19) pandemic. Even with the recent approval of the anti-viral drug, remdesivir, and Emergency Use Authorization of monoclonal antibodies against S protein, bamlanivimab and casirimab/imdevimab, efficient and safe COVID-19 vaccines are still desperately demanded not only to prevent its spread but also to restore social and economic activities via generating mass immunization. Recent Emergency Use Authorization of Pfizer and BioNTech's mRNA vaccine may provide a pathway forward, but monitoring of long-term immunity is still required, and diverse candidates are still under development. As the knowledge of SARS-CoV-2 pathogenesis and interactions with the immune system continues to evolve, a variety of drug candidates are under investigation and in clinical trials. Potential vaccines and therapeutics against COVID-19 include repurposed drugs, monoclonal antibodies, antiviral and antigenic proteins, peptides, and genetically engineered viruses. This paper reviews virology and immunology of SARS-CoV-2, alternative therapies for COVID-19 to vaccination, principles and design considerations in COVID-19 vaccine development, and the promises and roles of vaccine carriers in addressing the unique immunopathological challenges presented by the disease.

Contents

Abbreviations: APC, Antigen presenting cell; DC, dendritic cell DC; AD, adenovirus; AD, AAV, adeno-associated virus; LNPs, lipid nanoparticles; COVID-19, coronavirus disease 2019; ARDS, acute respiratory distress syndrome; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SARS, severe acute respiratory syndrome coronavirus; MERS, Middle East respiratory syndrome coronavirus; CDC, Centers for Disease Control and Prevention; WHO, World Health Organization; FDA, U.S Food and Drug Administration; EUA, Emergency Use Authorization; ACE2, angiotensin-converting enzyme 2; TMPRSS2, transmembrane protease serine; S protein, spike protein; N protein, nucleocapsid protein; E protein, Envelope protein; M protein, membrane protein; RBD, receptor binding domain; NTD, N-terminal binding domain; PPRs, pattern recognition receptors; NK cell, natural killer cell; RSV, respiratory syncytial virus; MSCs, mesenchymal stem cells; CTLs, cytotoxic T lymphocytes; ADE, antibody-dependent enhancement; Type 1 IFNs, Type 1 interferons; Toll-like receptor, TLR.

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1. Introduction

Human corona viruses were first discovered in the 1960s [1], being named after the crown-like structure of spike proteins on their surface that is critical to their infectivity. From current sequence databases, all human corona viruses have been traced to animal origins [2]. Some coronaviruses, HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1, are known to cause mild respiratory symptoms akin to the 'common cold' [3]. Severe acute respiratory syndrome coronavirus (SARS-CoV) and the Middle East respiratory syndrome coronavirus (MERS-CoV), which emerged in 2002 and 2012, respectively [4], are more infectious and responsible for life-threatening diseases in infants, older individuals, and immunocompromised patients.

A novel coronavirus, SARS-CoV-2, turned up in December 2019, followed by an outbreak first reported in Wuhan, China [5]. According to the Centers for Disease Control and Prevention (CDC) of the United States, transmission of the virus is primarily accomplished through direct contact or respiratory droplets [6] in a proximity and time-dependent manner, often requiring close contact within 6 feet over a period of 15 minutes or longer [7]. However, the possibility for airborne transmission under certain circumstances has recently been demonstrated [8–11], including prolonged exposure in an enclosed space without proper air handling [12–14]. The virus rapidly spread across the globe through travelers and the number of world-wide cases has now exceeded 72 million with over one million deaths as of December 14, 2020 [15]. It is recognized that the reported numbers are underestimates of actual infection cases, considering that infected but asymptomatic individuals were unlikely tested, and testing protocols and standardized reporting methods have been critically lacking [16]. The genetic sequence of SARS-CoV-2 was made available within weeks after its discovery and was identified as a betacoronavirus with close genetic similarity to SARS-CoV [17]. While SARS-CoV-2 is less deadly than SARS-CoV, it is transmitted much easier and faster [18], and the long incubation period and non-existent to moderate symptoms make the identification, tracing, and elimination of the disease caused by SARS-CoV-2 infection, coronavirus disease of 2019 (COVID-19), unexpectedly difficult [19]. The incubation period following the first exposure to SARS-CoV-2 is reported to be about 2-14 days and is likely to vary among age groups as well as individuals with comorbidities [20]. Similar to SARS-CoV and MERS-CoV, SARS-CoV-2 predominantly infects the airways, yielding symptoms and disease ranging from mild respiratory infections to severe acute respiratory syndrome, with the latter resulting in organ failure in some patients and eventually leading to death [21]. The most common COVID-19 symptoms are fever, dry cough, fatigue, and dyspnea, while severe symptoms are accompanied by systemic infection and pneumonia [22].

Over the past 100 years, vaccines have significantly increased life expectancy, fundamentally reshaping the community and economy [23]. As vaccination becomes widely available and used, the devastating impacts of many infectious diseases have faded [23]. Treatment of infectious diseases is costly as demonstrated by the seasonal flu which has a huge economic and societal burden for saving thousands of lives every year [24]. Widespread preventative vaccination can lower these costs and plays a pivotal role in protecting people from viral infections in an efficient and sustainable manner, resulting in complete elimination or significantly reduced transmission within the herd population [25]. Prevention strategies and therapeutic options against COVID-19, including convalescent plasma, monoclonal antibodies, repurposed drugs that are already approved in the clinic, and a variety of vaccines are being frantically explored by utilizing the advances in materials science approaches to delivery systems [26–31]. There are over 200 vaccine candidates being pursued globally; however, a lack of clarity in terms of the development of a safe and highly immunogenic COVID-19 vaccine remains (Fig. 1). The major hurdles in COVID-19 vaccine development include difficulty in validating and targeting the appropriate vaccine platform technologies, failure of generating long-term immunity, and inability to calm the cytokine storm. In addition to conventional vaccine forms of inactivated or live attenuated viruses, viral vectors, and subunit vaccines, emerging vaccine approaches using nanotechnology are highly adaptable and contribute to accelerated vaccine development [32]. However, most of these platforms have not been licensed for use in humans yet, leading to questions of long-term safety as well as the degree to which they can induce strong and long-term immunity [33]. An additional key concern is relying on the "S-only" vaccines, as mutations have been detected in the spike (S) protein of SARS-CoV-2 [34,35] and many candidate vaccines may need to be redesigned and tested. Historically, an ideal vaccine would be composed of an antigen or multiple antigens, adjuvant(s), and a delivery platform that can specifically be effective against the target infection, safe to a broad range of populations, and capable of inducing long-term immunity. This review outlines SARS-CoV-2 and the associated immune response with its infection, treatment options under development in the clinic, rationales and approaches to COVID-19 vaccine development, and promising roles of vaccine delivery systems in aiding the combat against the unprecedented pandemic.

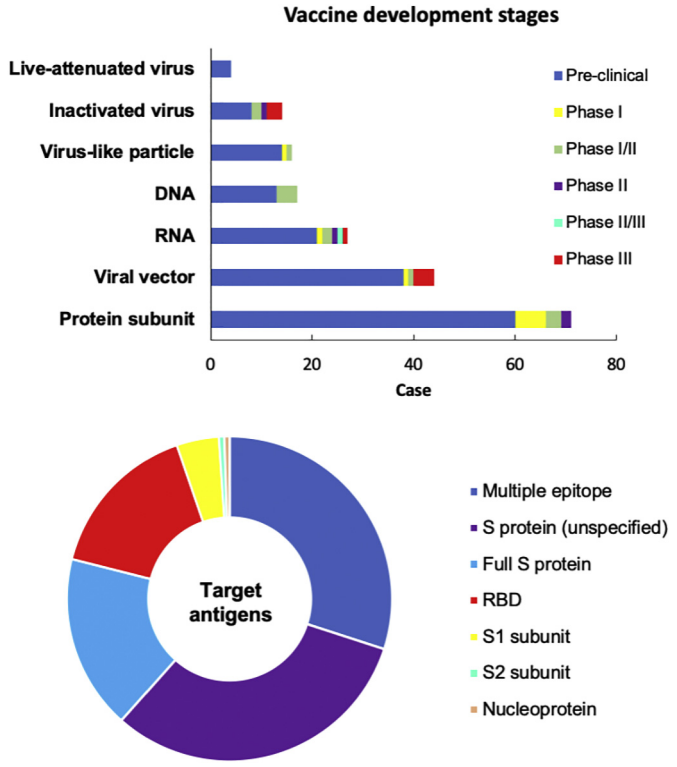

Fig. 1. Current pre-and clinical trials of COVID-19 vaccines, according to World Health Organization (WHO) as of December 8, 2020. Out of 214 vaccine candidates, 52 are in clinical trials and 162 are in pre-clinical studies. The lead vaccine candidates target the SARS-CoV-2 spike (S) protein including receptor binding domain (RBD) subunit, followed by full length S protein.

2. SARS-CoV-2 virology and vaccination against it: A brief overview

SARS-CoV-2 is an enveloped single stranded RNA (ssRNA) virus with spike-like-glycoproteins expressed on the surface forming a 'corona'. The SARS-CoV-2 genome shows 79.6% genetic identity to SARS-CoV and consists of four key proteins [17,36]. The S protein enables the attachment and entry of SARS-CoV-2 to the host cells, the membrane (M) protein is an integrity component of the viral membrane, and the nucleocapsid (N) protein binds to the viral RNA and supports the nucleocapsid formation, assisting in virus budding, RNA replication, and mRNA replication [37]. The envelope (E) protein is the least understood for its mechanism of action and structure, but seemingly plays roles in viral assembly, release, and pathogenesis [38] (Fig. 2). The S protein of the virus binds to the angiotensin-converting enzyme 2 (ACE2) receptor on the host cell surface, accompanied by being further primed by transmembrane protease serine (TMPRSS2) [39–41]. TMPRSS2 cleaves the S protein into two subunits, S1 and S2, during viral entry into the host cell via membrane fusion. ACE2 expression is ubiquitous in the nasal epithelium, lung, heart, kidney, and intestine, but it is rarely expressed in immune cells [42,43]. Recent studies have shown that there are other receptors involved in viral entry in different cell types. As in the case of SARS-CoV, CD-147 on the epithelial cells is found to be a receptor for SARS-CoV-2 as well [44]. CD26 (dipeptidyl peptidase 4, DPP4), originally discovered during the cellular entry of MERS-CoV, has also recently emerged as a potential receptor for SARS-CoV-2 [45,46] and structural analysis showed SARS-CoV-2 S protein interaction with CD26 of the host cells [47].

The critical role that the S protein plays in viral entry makes it an attractive target for COVID-19 vaccines [48]. The S1 subunit contains the profusion-state of the receptor binding domain (RBD) responsible for binding to ACE2, while the S2 subunit contains the cleavage site that is critical for the fusion of viral and cellular membranes [49]. Computational analyses and knowledge previously gained from SARS-CoV and MERS-CoV identified the full-length S protein, S1, RBD, and S2 subunit proteins to be key epitopes for inducing neutralizing antibodies [5,50]. While structurally similar, the SARS-CoV-2 S protein has shown 20 times higher binding affinity to host cells than SARS-CoV S protein, explaining the high transmission rate of COVID-19 [4]. The S protein in both SARS-CoV and SARS-CoV-2 additionally induces the fusion between infected and non-infected cells, allowing for direct viral spread between cells while avoiding virus-neutralizing antibodies. The possibility of utilizing multiple neutralizing epitopes makes the S protein the most popular target for vaccination. In particular, the S1 epitope containing both the N-terminal binding domain (NTD) and RBD has been used in vaccine development, and especially the antibodies against the RBD domain have previously demonstrated to prevent infections by SARS-CoV and MERS-CoV [51,52]. The N protein is the most abundant protein among coronaviruses with a high level of conservancy. While patients have shown to develop antibodies against the N protein [53], its use in vaccination remains controversial. Some studies demonstrated

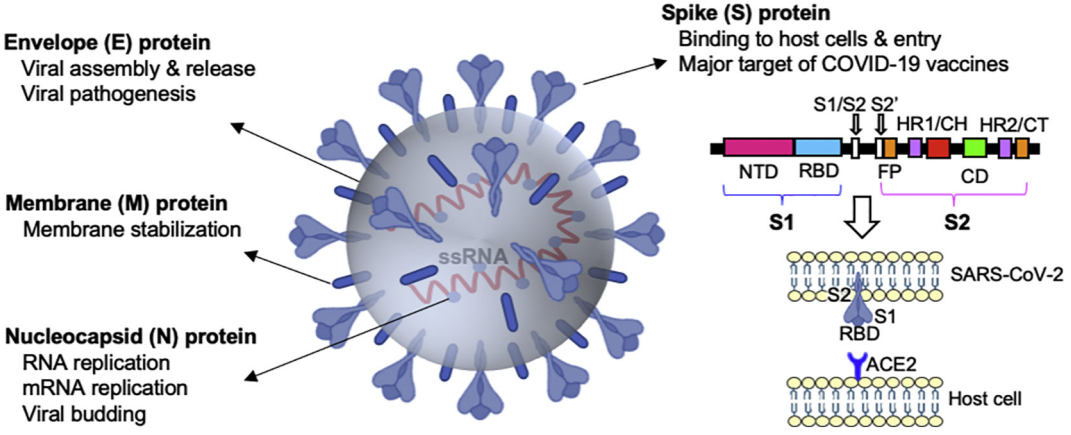

Fig. 2. Structure of SARS-CoV-2 with a positive-sense, single stranded RNA that shares 79.6% sequence identity to SARS-CoV and encodes the S, M, E and N proteins. The S protein, a major target for vaccination, contains three critical elements: S1, receptor binding domain (RBD), and S2 subunits. The S1 subunit contains the N-terminal and the C-terminal subdomain (CTD) that are in a closed conformation until specific proteases cleave the S1/S2 and S2' sites. The RBD is located in the S1-CTD and necessary for binding to the ACE2 receptor on the surface of the host cells. The S2 subunit domain forms a trimeric structure and contains the fusion peptide (FP) and two heptad repeats (HR1 and HR2) which are required for the fusion of viral and host membrane. The most abundant N protein packages the genome into a virion for effective viral replication, the M protein is involved in viral integration, and the E protein facilitates assembly, envelope formation, and budding.

strong N-specific humoral and cellular immune responses [54], while others showed insignificant contribution of the N protein to production of neutralizing antibodies [55]. Immunization with the M protein, a major protein on the surface of SARS-CoV-2, elicited efficient neutralizing antibodies in SARS patients [56]. Structural analysis of the transmembrane portion of the M protein showed a T cell epitope cluster that enables the induction of strong cellular immune response against SARS-CoV [57], and it could also be a useful antigen in the development of SARS-CoV-2 vaccine. As compared to the S, N, and M proteins, E proteins of SARS-CoV-2 are not promising for vaccination as their structure and low quantity is unlikely to induce an immune response [58].

3. COVID-19 pathology and immune response to SARS-CoV-2

The lungs are exposed to thousands of liters of air daily, creating vast opportunities for airborne pathogens to enter the body [59]. Therefore, the immune system within the lungs has evolved to be highly sensitive and constantly active [60–62]. Mucus, a protective barrier in the lungs, coats the epithelial layers and entraps small particles and pathogens which are easily cleared from the body by coughing [63]. However, respiratory viruses such as coronavirus are able to permeate through this barrier. The virus infects the lung cells and triggers an immune response by recruiting cells that release inflammatory cytokines and prime T and B cells for immune response [64]. This process is intended for viral clearance; however, in some cases dysfunctional immune response occurs, causing severe damage to the lungs and eventually leading to systemic inflammation. Knowledge of the host immune response to SARS-CoV-2 is still not fully understood despite continuing research. However, clinical data obtained from SARS-CoV and MERS-CoV, allows for some fundamental understanding and prediction of how the immune system will respond [65].

Upon inhalation, lung epithelial cells recognize SARS-CoV-2 as a pathogen via pattern recognition receptors (PRRs) and secrete molecules to recruit immune cells that mediate innate and adaptive immunity [66]. First responders are alveolar macrophages bearing both inflammatory TLR2, 4, 6, IL-1R, IFNγ-R, and TNFR, as well as CD200R, SIRP, mannose receptor, TREM2, IL-10R, and TGFBR that play regulatory roles in controlling immune response [67]. A subset of airway- and lung- residing $CD103^+$ dendritic cells (DCs) use their dendritic structures to permeate through the epithelial layer for antigen capture [68,69]. Antigen is subsequently processed for presentation by MHC I and MHC II to $CD8^+$ T and $CD4^+$ T cells, respectively [70]. $CD4^+$ T cells assist in the overall adaptive response by stimulating B cells and $CD8^+$ T cells, mediating both durable antibody-mediated and cellular immune responses and development of memory cell populations [71]. DCs also migrate to the lymph nodes for naïve T cell education [72]. In particular, Th1 type immune response plays a dominant role in adaptive immune response to viral infections [73]. $CD4^+$ T cells are rapidly activated into GM-CSF-secreting T helper cells (Th1) cells, which activate the $CD14^+$ $CD16^+$ monocytes with high IL-6 expression for accelerated inflammation [74]. Th17 cells produce IL-17 to further recruit monocytes, macrophages, and neutrophils and stimulate cytokines including IL-1β, IL-6, and IL-1 amongst others [75,76].

SARS-CoV-2 S protein binds to epithelial cells in the nasal cavity via ACE2 receptors [77] Upon entry, intracellular sensors, TLR7/8 and RIG-I/MDA-5, signal for the activation of transcription factors, IRF3/7 and NF-κB, and production of type I interferons (IFNs) and inflammatory cytokines [78]. Type I IFNs play a critical role in the adaptive immune response and preventing viral spread by stimulating other immune cells for production of inflammatory cytokines, chemokines, and anti-viral enzymes [79,80]. However, like SARS-CoV and MERS-CoV [81], SARS-CoV-2 can evade type I IFNs by avoidance [82,83], suppressed IFN induction [84–88], and suppressed IFN signaling [89,90]. Recently, this evasion process has proven to be a major underlying factor in the development of severe COVID-19 cases [91] and could be an important target for therapeutic intervention.

Within 1-2 days post-infection, SARS-CoV-2 locally replicates, allowing detection by RT-PCR from a nasal swab [92]. Many patients at this stage are often asymptomatic, but still highly infectious. Notably, viral load of greater than 5-6 copies/mL is an independent predictor of disease progression and mortality [93]. In the following 3-7 days, SARS-CoV-2 remains restricted to the upper respiratory airway in the majority of patients with mild symptoms that can be monitored and managed from home [94]. However, for some patients, the virus continues to spread into the respiratory tract and lungs with clinical manifestation [95]. SARS-CoV-2 progresses to the gas exchange portions of the lung and infects alveolar cells [93]. Viral spread worsens and speeds as the virus propagates in infected cells, being released upon lysis and further damaging the epithelium to allow for tissue permeation. Cellular machinery presents viral peptides on MHC I to $CD8^+$ T cells [70], leading to clonal expansion and development of SARS-CoV-2-specific memory cells. $CD8^+$ T cells further assist in clearing infected cells through induction of apoptosis via perforin and granzymes [96]. Responding inflammatory macrophages, monocytes, neutrophils, and lymphocytes struggle to combat the virus [97,98]. Simultaneously, the deadly levels of inflammatory IFNα, IFNγ, IL-1β, IL-6, IL-12, IL-18, IL-33, TNFα, TGFβ, CCL2, CCL3, CCL5, CXCL8, CXCL9, CXCL10, and more are released, contributing to a cytokine storm. In the late stages of infection by coronaviruses including SARS-CoV, MERS-CoV, and SARS-CoV-2, cytokine storms are a major cause of disease progression, acute respiratory distress syndrome (ARDS), systemic inflammation, multiple organ failure, and eventually leading to death [99–101] (Fig. 3). The anti-viral immune response is crucial for viral clearance at early stages of infection, but a robust and persistent immune response can lead to massive cytokine production and damage to host tissues [102,103]. Approximately 14% of COVID-19 patients become severely ill with dyspnea and difficulty breathing, and 5% enter critical conditions including respiratory failure, septic shock, and multiple organ dysfunction and failure [95]. The first autopsy of a COVID-19 patient revealed accumulation of mononuclear monocytes and inflammatory T cells in the lungs but with low levels of active T cells against SARS-CoV-2 [97]. In other severe COVID-19 cases, distinct profiles of cell populations have been observed [104]. Patients showed similar overall levels in total activated T cells and cytotoxic T lymphocytes (CTLs), particularly increased levels of $CD4^+$ T cells, leukocytes, neutrophils, and decreased monocytes, eosinophils, basophils, Treg, memory and, $CD8^+$ T cells [105,106]. Additional studies found increased concentrations of IL-1β, IFN-γ [107], and IL-6 [108,109]. Upon recognition of a pathogenic antigen and recovery, a subset of memory T cells remain in the lungs [110], ready for action upon secondary exposure or infection. Resident memory T cells were proven to be critical for viral clearance as a human respiratory syncytial virus (RSV) study showed that they could eradicate the virus without assistance of antibodies [111].

The transition between innate and adaptive immune responses is crucial for controlling SARS-CoV-2 infection and is dependent on $CD4^+$ T cells to interact with B cells to produce specific neutralizing antibodies [112]. Previous studies have reported specific neutralizing antibodies against the S protein [113–115]. However, there are still several questions regarding the significance of the antibodies to the viral protein and the cross-reactivity of antibodies to other prevalent coronaviruses, in particular between SARS-CoV and SARS-CoV-2, with 79.6% identical structural features [116,117]. Antibody responses are detectable within 1-2 weeks after symptom onset in most infected individuals [118]. Specifically, IgM and IgA against SARS-CoV-2 have been detected within the 1st week of infection, while IgG antibodies have been detected around day 14 after the onset of symptoms [119–122]. Recent studies indicate that neutralizing antibodies were closely correlated with the severity in COVID-19 patients [123], and antibody responses faded within weeks after infection [124]. Furthermore, the neutralizing antibody response in asymptomatic individuals decreased faster and remained lower than in symptomatic individuals [125]. In the case of SARS-CoV, long lasting, specific IgG, and neutralizing

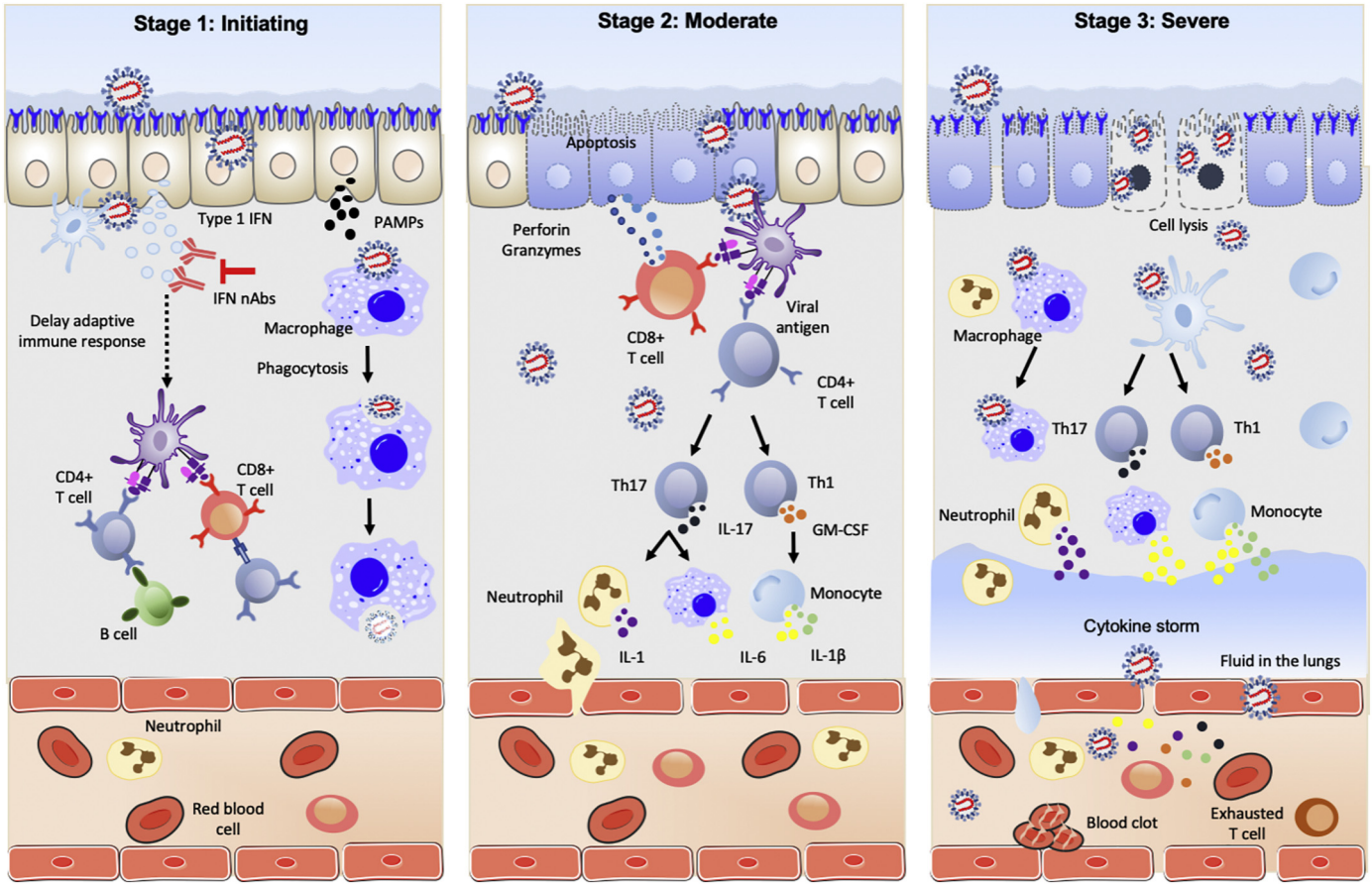

Fig. 3. Immune response to SARS-CoV-2 at varying pathological stages. In stage 1, in the nasal cavity, SARS-CoV-2 binds to ACE2 receptors on epithelial cells by the S protein, enters the cell, and starts to replicate. The virus proliferates and simultaneously travels down the respiratory tract, and clinical manifestation of symptoms start appearing. Upon infection, the virus activates type I IFNs. In COVID-19 patients, type 1 IFN production is delayed by SARS-CoV-2, which lowers the adaptive immune response. The host innate immune system detects SARS-CoV-2 via recognition of pathogen-associated molecular patterns (PAMPs) by the alveolar macrophages activating cytokines such IL-6 and TNF-α leading to phagocytosis of the virus or activation. In stage 2, infected epithelial cells present viral antigens to both $CD4^+$ and $CD8^+$ T cells. $CD8^+$ T cells release perforins and granzymes that induce apoptosis of the infected cells. $CD4^+$ T cells rapidly activate to become Th1 cells that secrete GM-CSF and further induce monocytes by high IL-6 levels. An increase in monocyte subpopulation promotes IL-1β production. Th17 cells produce IL-17 to further recruit monocytes, macrophages, and neutrophils, and stimulates other inflammatory cytokines such as IL-6, IL-1, and IL-1β. In stage 3, inflammatory cells release additional cytokines which amplifies the cytokine storm and exacerbates the systemic inflammatory response, eventually leading to ARDS, multiorgan failure, and death.

antibodies were reported to last as long as 2 years after infection [126], while MERS-CoV neutralizing antibodies were detected within 14-21 days of illness onset [127]. No correlation between protections from SARS-CoV-2 and other coronaviruses have been confirmed, and it is unknown what neutralizing antibody titers are necessary to provide full protection [128]. Establishing such correlations could help provide insightful information about COVID-19 vaccination.

4. Non-vaccination treatment options for COVID-19

The similarities found between SARS-CoV-2 with SARS-CoV and MERS-CoV have resulted in the rapid understanding of its pathology [129] and current efforts are centered in accelerating the development of therapeutics for COVID-19 by repurposing drugs that are already clinically available, incorporating new technologies, or reinforcing the immune system [130]. Repurposing antiviral drugs that are FDA-approved or currently under investigation for other viral infections has been a popular approach to the development of COVID-19 therapy for its immediate availability in the clinic [131], and recently, veklury (remdesivir), was the first drug approved by the FDA for the treatment of COVID-19 followed by Emergency Use Authorization (EUA) of monoclonal antibodies, bamlanivimab and casirimab/imdevimab (Table 1). The antiviral and chemotherapeutic agents used in the current clinical trials directly act on COVID-19 limiting the viral replication in host cells. Another approach aims to modulate the immune system to be boosted using cell-based therapy and convalescent plasma treatment, or inhibition of inflammatory processes linked with cytokine storms (Fig. 4). These treatments are targeted to achieve lowered SARS-CoV-2 burden in patients, alleviate severe symptoms, and slow the progress of COVID-19 [132].

4.1. RNA polymerase inhibitors

Remdesivir, originally developed for Ebola infections, was the first anti-viral drug approved by the FDA for COVID-19 therapy on October 22, 2020 [133,134]. It terminates RNA synthesis and inhibits SARS-CoV-2 genome replication, which previously exhibited antiviral activities against SARS-CoV and MERS-CoV [135,136]. Clinical trials of remdesivir on randomized, placebo-controlled, treatment groups with 1,062 patients improved the recovery time of some COVID-19 patients at advanced stages [137]. Favipiravir, a guanine analogue, inhibits RNA polymerase and is currently used for influenza treatment [138]. A combination of favipiravir and interferon-α (ChiCTR2000029600) or baloxavir marboxil (ChiCTR2000029544) showed anti-SARS-CoV activity in Vero E6 cells, and randomized clinical trials to evaluate their safety and efficacy are underway [139].

Table 1
Repurposed drugs for COVID-19.

Category	Drugs	Mechanism of action	Status of clinical use
Anti-viral	Remdesivir	Inhibition of viral replication	Approved by the FDA
	Favipiravir	Inhibition of viral-RNA dependent RNA polymerase	Under clinical trials
	Lopinavir-Ritonavir	Inhibition of protease enzymes (HIV reverse transcriptase inhibitors)	Under clinical trials
	Umifenovir	Inhibition of viral and cellular membrane fusion	Under clinical trials
	Camostat (TMPRSS2 inhibitor)	Blockade of viral maturation and entry to host cells	Under clinical trials
	Hydroxychloroquine	Inhibition of virus entry, elevate endosomal pH and interfere with ACE2 glycosylation	Emergency use terminated by the FDA (Serious cardiac events)
	Azithromycin	Indirect immunomodulatory effects	Under clinical trials
	Tocilizumab	Blockade of IL-6 receptors and its downstream signaling pathways	Under clinical trials
	Anakinra	Blockade of IL-1 receptors and its downstream signaling pathways	Under clinical trials
Anti-inflammatory	Ruxolitinib	JAK signaling inhibition, Immune suppression	Under clinical trials
	Baricitinib	Inhibition of viral invasion and JAK signaling, Immune suppression	Under clinical trials
	Thalidomide	Reduction of inflammatory cell infiltration, reduce cytokine storm	Under clinical trials
	Glucocorticoids	Suppression of immune and inflammatory response	Dexamethasone authorized use in critically ill patients
Monoclonal antibody	Bamlanivimab	Inhibit viral entry into host cells	Emergency Use Authorization
	Casirivimab and imdevimab	Inhibit viral entry into host cells	Emergency Use Authorization
Plasma therapy	Convalescent plasma	Virus elimination via virus-specific antibodies	Under clinical trials
Cell-based therapy	Mesenchymal stem cell	Facilitate tissue regeneration and immune suppression	Under clinical trials
	NK cell	Strengthen immune response	Under clinical trials

Other antivirals currently repurposed for COVID-19 therapy include neuraminidase inhibitor oseltamivir. Oseltamivir has been used for influenza A and B viruses as they require neuraminidase for virus release to host cells. SARS-CoV-2 does not express neuraminidase therefore combination therapy of oseltamivir together with protease inhibitors has shown efficacy [140,141].

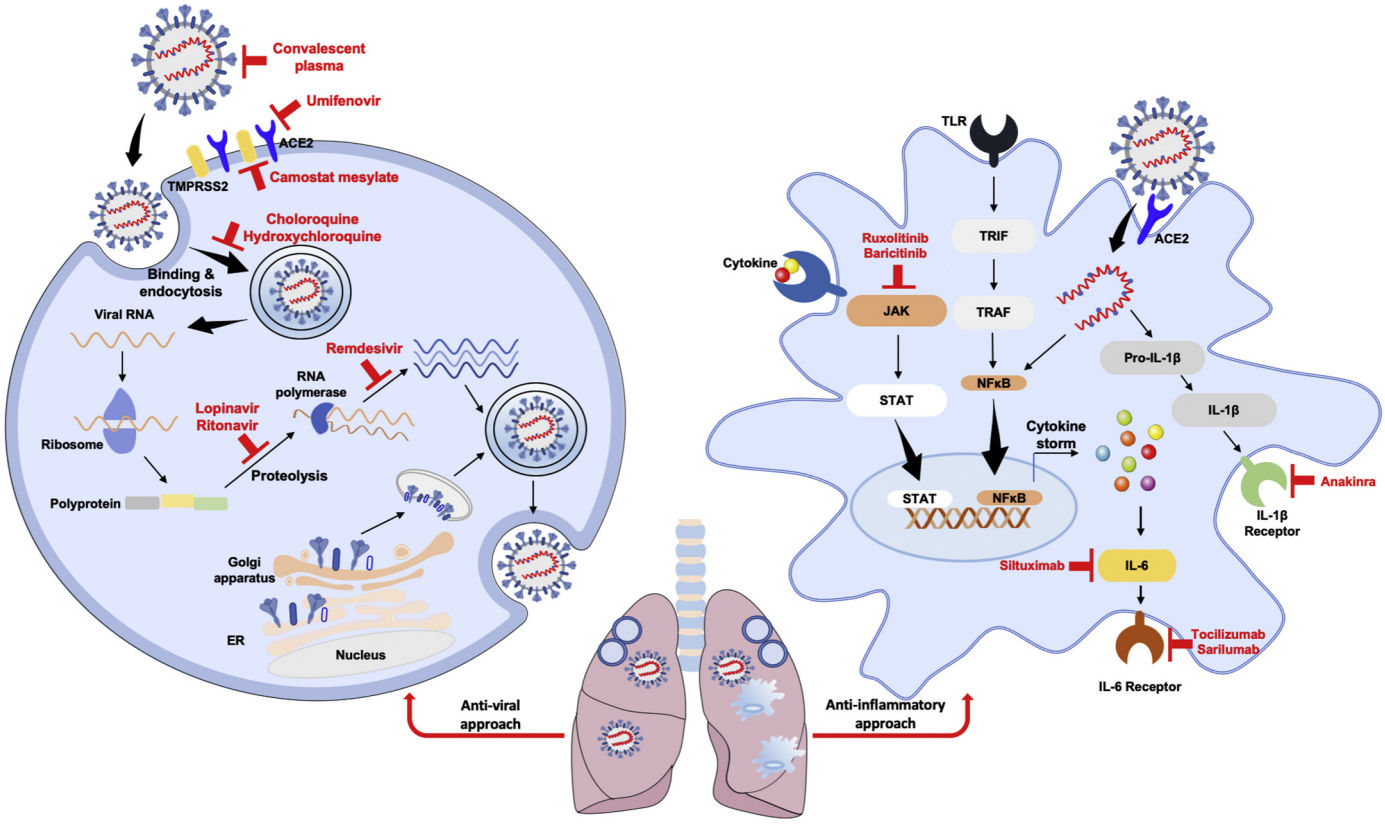

Fig. 4. Schematic representation of SARS-CoV-2 infection and signaling pathways as potential targets for treatment. SARS-CoV-2 binds to ACE2 and TMPRSS2 receptors through the S protein resulting in viral-host membrane fusion. Umifenovir and camostat mesylate inhibit the fusion process of viral entry. Virus enters the cells by endocytosis, and chloroquine and hydroxychloroquine inhibit the endosomal acidification and interfere with glycosylation of ACE2. The viral RNA is released and translated to essential viral polyproteins, 3CLpro and PLpro. Lopinavir/Ritonavir inhibits the activity of 3CLpro. Viral replication requires the RNA polymerase and remdesivir incorporates into nascent viral RNA causing RNA synthesis arrest, preventing viral replication. SARS-CoV-2 infection induces a massive release of cytokines including IL6, IL-1β, and IL-1 and increases the activation of NF-κB and JAK-STAT signaling pathways. Tocilizumab and sarilumab bind to membrane and soluble IL-6 receptors suppressing the JAK-STAT signaling pathway and attenuating inflammatory processes. Baricitinib and ruxolitinib inhibit the kinase activities of JAK1 and JAK2 and anakinra, an IL-1 receptor antagonist, reduces hyperinflammation and respiratory distress.

4.2. Protease inhibitors

Lopinavir (LPV) and ritonavir (RTV) are protease inhibitors approved for treating HIV-1 infection [142]. Due to its poor oral bioavailability, LPV is co-formulated with RTV for increased drug solubility and improved anti-viral activity [142]. LPV and RTV were initially hypothesized to inhibit 3-chymotrypsin-like protease of SARS-CoV and MERS-CoV, implicating their potential use for COVID-19 treatment [143,144]. LPV and RTV have been used as a triple combination therapy with either PEG-IFN-α and ribavirin [145] or chloroquine/hydroxychloroquine in COVID-19 treatment [146]. Some clinical trials have shown that LPV/RTV did not help inhibit SARS-CoV-2 proteases (ChiCTR2000029308). Other protease inhibitors repurposed for COVID-19 clinical trials are RTV in combination with ASC09 and darunavir, an anti-viral drug purposed for treatment of HIV/AIDS (NCT04261907)

4.3. Virus-cell fusion inhibitors

SARS-CoV-2 uses the ACE2 receptor, another common target in COVID-19 clinical trials [147], to enter the host cell. A recent *in vitro* study showed that soluble human recombinant ACE2 (hrsACE2) drastically reduced SARS-CoV-2 loads in Vero cells in a dose-dependent manner [148]. Arbidol, also known as umifenovir, blocks the virus-cell membrane fusion as well as fusion of viral membrane with the endosome after endocytosis [149]. In H1N1 influenza viral infection, it directly interacts with the virus to stabilize hemagglutinin (HA) [150]. In SARS-CoV-2, umifenovir interfered with virus binding and intracellular vesicle trafficking in Vero E6 cells [149], and is also being considered as a COVID-19 therapy in combination with protease inhibitors [151].

Well-known anti-malarial and anti-autoimmune agents, chloroquine, and hydroxychloroquine block viral infection by increasing endosomal pH to inhibit the membrane fusion between virus and host human cells [152,153]. Hydroxychloroquine, a derivative of chloroquine, has similar pharmacokinetic effects to chloroquine with fewer side effects [154]. Hydroxychloroquine treatment showed viral clearance in COVID-19 patients and combination therapy with azithromycin elevated the anti-viral effect [155]. Additionally, a number of clinical trials in China showed marginal therapeutic efficacy of hydroxychloroquine in patients with COVID-19-induced pneumonia [156]. However, the FDA has warned about using chloroquine/hydroxychloroquine outside the hospital due to its serious arrhythmia risks in COVID-19 patients with a history of hypertension, heart failure, and chronic kidney diseases, rejecting approval for COVID-19 therapy [157]. Chloroquine treatment showed specific inhibition of SARS-CoV replication by interfering with the glycosylation of ACE2 receptor [153], effectively reducing the copy number of SARS-CoV-2 [158]. Interestingly, ACE2 receptor inhibitors were shown to increase the expression of ACE2 receptors, resulting in enhanced viral entry, limiting their clinical benefits [159].

4.4. Anti-inflammatory agents

Several immune-modulating drugs that regulate inflammation are being tested for their efficacy for severe COVID-19 [160]. The cytokine storm is a critical determinant of mortality in patients with serious symptoms in the late stage of COVID-19 [103]. IL-6 is a key inflammatory cytokine which is highly elevated in severely ill COVID-19 patients [76,161] and the IL6/IL-6 receptor (IL-6R) signaling pathway is a promising target for alleviated inflammatory symptoms. Tocilizumab, a humanized anti IL-6 receptor antibody, approved for used in rheumatoid arthritis [162] was shown to be effective against cytokine release syndrome associated with CAR-T cell therapy [163]. Tocilizumab binds to both membrane and soluble IL-6 receptors in suppressing the JAK-STAT signaling pathway and downregulating the downstream inflammatory molecules [164]. Administration of tocilizumab has shown improvement of respiratory function in COVID-19 patients within a few days [164]. Sarilumab, another IL-6 receptor blocker, also inhibits the IL-6 signaling pathway and a clinical trial is underway to evaluate its safety and efficacy in COVID-19 patients with serious complications [165]. Inhibition of JAK-STAT pathways using JAK inhibitors approved for rheumatoid arthritis and psoriatic arthritis therapy is another potential immunotherapeutic approach to alleviating the cytokine storm in COVID-19 [166]. Baricitinib inhibits the kinase activities of JAK1 and JAK2 and efficiently reduced SARS-CoV-2 viral infection [167], and rituxolitinib, another JAK1 and JAK2 inhibitor, has shown some efficacy in reducing severe respiratory distress [168]. IL-1 was reported to be increased in some COVID-19 patients [5] and blocking IL-1 using anakinra, an IL-1 receptor antagonist previously approved for rheumatoid arthritis therapy, has shown promising results in reducing hyperinflammation and respiratory distress in COVID-19 patients (NCT04324021).

Corticosteroids appear to be beneficial for critically ill COVID-19 patients according to a meta-analysis of seven randomized clinical trials that included 1,703 patients recruited from 5 different continents [169]. Steroids are cheap, readily available, and are effective in reducing deaths of the most severely affected COVID-19 patients [169]. Dexamethasone, an anti-inflammatory drug, has anti-fibrotic and vasoconstrictive effects [170] and many clinical trials in the past decade have tested its efficacy in treating pneumonia, sepsis, or ARDS [171]. In ARDS patients, corticosteroids improved oxygen saturation and reduced inflammation [172]. Since a recent trial showed dexamethasone reduced the death of ventilated COVID-19 patients by one-third [173], steroids have been proposed as potential COVID-19 drugs, attributed to their ability to help dampen inflammation and other immune system responses [174]. Despite the benefit of calming the cytokine storm, suppressed inflammation and immune response impede viral clearance and prevention of viral spread.

4.5. Cell-based immunotherapy

Natural Killer (NK) cells are innate immune responders and critical for viral clearance and immunomodulation. In addition to their antiviral activities, NK cells also play key immunopathological roles during the infections of RSV, influenza A virus, and hepatitis B virus [175]. A previous study showed that migration of NK cells and macrophages helped clear SARS-CoV [176], and NK cells from COVID-19 patients showed increased perforin levels, providing a basis for understanding the role of NK cells in COVID-19 infections [177]. Given the importance of NK cells in acute viral infection, recent studies have demonstrated potential strategies for NK cell-based immunotherapy for COVID-19 [178]. An ongoing clinical trial in China investigates the use of NK cells for viral clearance in severe COVID-19-associated pneumonia patients (NCT04280224). Self-renewing, multi-potent mesenchymal stem cells (MSCs) also play an important role in inflammatory diseases with their anti-inflammatory properties [179]. MSCs have been shown to help alleviate the cytokine storm, repair pulmonary epithelial damage, and facilitate alveolar fluid clearance [180]. Animal studies using MSCs demonstrated a reduction of influenza A H5N1 acute lung injury [181]. Several phase I and II clinical trials have confirmed the safety and efficacy of MSC therapy for ARDS patients [182,183].

4.6. Monoclonal antibodies and convalescent plasma therapy

Antibody-mediated humoral immunity is critical for the prevention of recurring viral infections, and neutralizing antibodies are specific and effective to target the virus. This classical adaptive prevention approach has been applied to many infectious diseases including SARS-CoV, MERS-CoV, and the H1N1 infections [184]. The SARS-CoV-2 S protein is the major inducer of neutralizing antibodies and monoclonal antibodies against it, bamlanivimab [185], casirivimab and imdevimab [186] were approved for emergency use authorization (EUA) by the FDA on November, 2020. Other monoclonal antibodies against S protein also

include 80R [187], CR3014 [188], CR3022 [189], and m396 [190] are under development. CR3022 is currently being tested for binding affinity to the RBD of SARS-CoV-2 S protein and showed neutralization with CR3014 combination therapy [191], and 80R and m396 also showed potent cross-reactive neutralization in SARS-CoV [192]. Four human-original monoclonal antibodies (B5, B38, H2 and H4) from a convalescent patient showed binding to the SARS-CoV-2 RBD resulting in high neutralization [192–194]. Some studies have shown a lower mortality rate among the patients treated with convalescent plasma, compared with placebo [195]. However, treatment with convalescent plasma is limited as the therapy must be transfused, and there have been adverse effects including mild fever and allergic reactions [196].

5. COVID-19 vaccines

5.1. Vaccination strategy

The world has been in varying states of lockdown since March 2020, causing profound economic and social consequences. In addition, despite implementation of masks and other safety protocols, a number of lives have been lost to COVID-19. COVID-19 quickly and unexpectedly spread internationally, with millions worldwide already exposed to the virus. Some estimates indicate that between 1-20% of the population have been exposed to COVID-19; however, these estimates are questionable as many patients are asymptomatic and go untested [16]. While a few countries have considered slow and intentional exposure, akin to chicken pox parties from the 1980's, this would need to be done safely without overburdening hospitals and would take years [95]. Additionally, SARS-CoV-2 is much more deadly than the chicken pox or influenza viruses and has shown to cause long-lasting effects on the lungs, the heart, and the central nervous system, which are still not fully understood [197]. A vaccine is desperately needed not only for individual healthcare but also to achieve herd immunity, in which at least 70% of the total population would need to be vaccinated [198]. Unfortunately, immune response to a pathogen is often heterogeneous, and varies between individuals based on age, environment, and underlying health conditions [199,200]. While vaccines that effectively generate specific and controlled responses can allow for achieving herd immunity, chances for infections and recurring infections of SARS-CoV-2 [201] necessitate therapeutic interventions and combination therapy. Preventative vaccination is the safest and most cost-effective way to prevent COVID-19 illness and death, and the best option to combat anticipated future variants. Annually, the CDC performs surveillance on circulating strains of the influenza virus and adjusts protocols to reflect the findings in preparation of vaccines against the most prevalent strains in the coming year [202]. A similar global strategy is warranted to prevent or minimize COVID-19 and future SARS-CoV-2 variants prevalence.

5.2. Immunity generated by COVID-19 vaccines

COVID-19 vaccines are in three broadly categorized forms of molecular, particular, and cell-based vaccines, sharing the same goal of stimulating the immune system against SARS-CoV-2 with the generation of memory cells. Molecular vaccines use whole proteins, fragmented peptides, or whole viruses and generate immune response with the aid of antigen presenting cells (APCs) such as DCs [203]. In particular, DCs play pivotal roles in capturing molecules, fragmenting them into smaller peptides, and presenting the antigenic peptides on their MHC I and II (or HLAs in humans) to prime T cells for the initiation of cellular and humoral immunity against the virus [204]. Antigen presentation by MHC I, along with co-stimulatory surface molecules such as CD80 and CD86 on DCs, leads to $CD8^+$ T cell activation. Antigen presentation by MHC II primes $CD4^+$ T cells which can both stimulate B cells to produce neutralizing antibodies against the pathogen and additionally assist in activation of CTLs [205]. Virus and virus-infected cells are rapidly recognized and cleared by efficiently activated immunity initiated by APCs and in collaboration with T cells and other immune cells.

Most viral vaccines primarily aim to generate antibody-mediated immune response, and emerging data on T cell immunity in COVID-19 patients can guide in developing strategies for effective protection from SARS-CoV-2 [206]. Memory $CD4^+$ T and $CD8^+$ T cells primed against the S, N, and M proteins were detected in 100% and 70% of recovered COVID-19 patients, respectively [207,208], while immunization with SARS-CoV peptide-presenting DCs generated a higher number of virus-specific $CD4^+$ T and $CD8^+$ T cells and increased overall survival [209]. Although T cells are important for efficient vaccination, several SARS-CoV vaccine formulations showed increased immunopathological signs rather than protection [210,211], requiring further studies on T cell- mediated immunity by COVID-19 vaccination.

Neutralizing antibody responses against SARS-CoV-2 S and N proteins have been detected in most patients within 3 weeks [212]. However, recent studies showed that $CD4^+$ T cells in healthy individuals who have not been exposed to SARS-CoV-2 also recognized SARS-CoV-2 proteins, raising a concern of pre-existing immunity possibly contributing to antibody-dependent enhancement (ADE) [213] therefore lowering B-cell stimulation. ADE is a potential challenge in the development of an effective COVID-19 vaccine, particularly via enhanced T cell-mediated immunity. When ferrets were vaccinated with modified vaccinia Ankara vaccine (MVA) that was expressing SARS-CoV S protein, increased infection by the virus [214] along with antibodies against the S protein and induced lung injury were observed in association with ADE [215].

5.3. Considerations in COVID-19 vaccine design and administration

Efficient and safe COVID-19 vaccines will need to generate desired humoral and cellular immunity against SARS-CoV-2, while simultaneously minimizing adverse side effects such as cytokine storms, and also allowing for rapid development and deployment, particularly for anticipated viral mutations. Therefore, multi-dimensional considerations of appropriate antigen selection, formulations, administration routes, and vaccination timing need to be made.

5.3.1. Antigen selection for desired immune response

Independent of the vaccination platform, selecting the most potent target antigen(s) is pivotal in the development of effective COVID-19 vaccines [216,217]. Ideal antigens should be specific to SARS-CoV-2 and readily recognized by the immune system (Fig. 5).

5.3.1.1. Antibody production. The RBD of the S protein is a prime target for neutralizing antibody-mediated inactivation of SARS-CoV-2 in preventing viral entry into host cells by blocking viral binding to ACE2 receptors, limiting its propagation, and spread. The antibodies generated by a COVID-19 vaccine must not only bind to SARS-CoV-2, but neutralize viral functions, while avoiding the possibility of ADE-assisted viral entry as observed in SARS patients [218]. Similar to the SARS-CoV S protein that generated neutralizing antibodies in mouse models [55,219], convalescent plasma from recovered SARS-CoV-2 patients contained neutralizing antibodies specific to the S protein (full length S, S1, RBD, and S2) [114,220,221]. Consequently, most COVID-19 vaccines almost exclusively utilize the SARS-CoV-2 S antigen which is prone to mutation [119], including G614 and D641 [35], although insignificant to impact on current clinical trajectories. Although the G641 mutation is sensitive to neutralization in convalescent sera, it seems to be more infectious than the D641 mutation [222,223]. The S protein is highly glycosylated and modified glycosylation sites have been investigated to assess the infectivity and reactivity to neutralizing antibodies [119]. For example, deletion of the heavily glycosylated residues, such as N331 and N343, reduced infectivity of SARS-CoV-2, and other modifications in the RBD, A475V, L452R, V483A, and F490L, resulted in resistance to neutralizing antibodies [119]. The observation that prefusion-

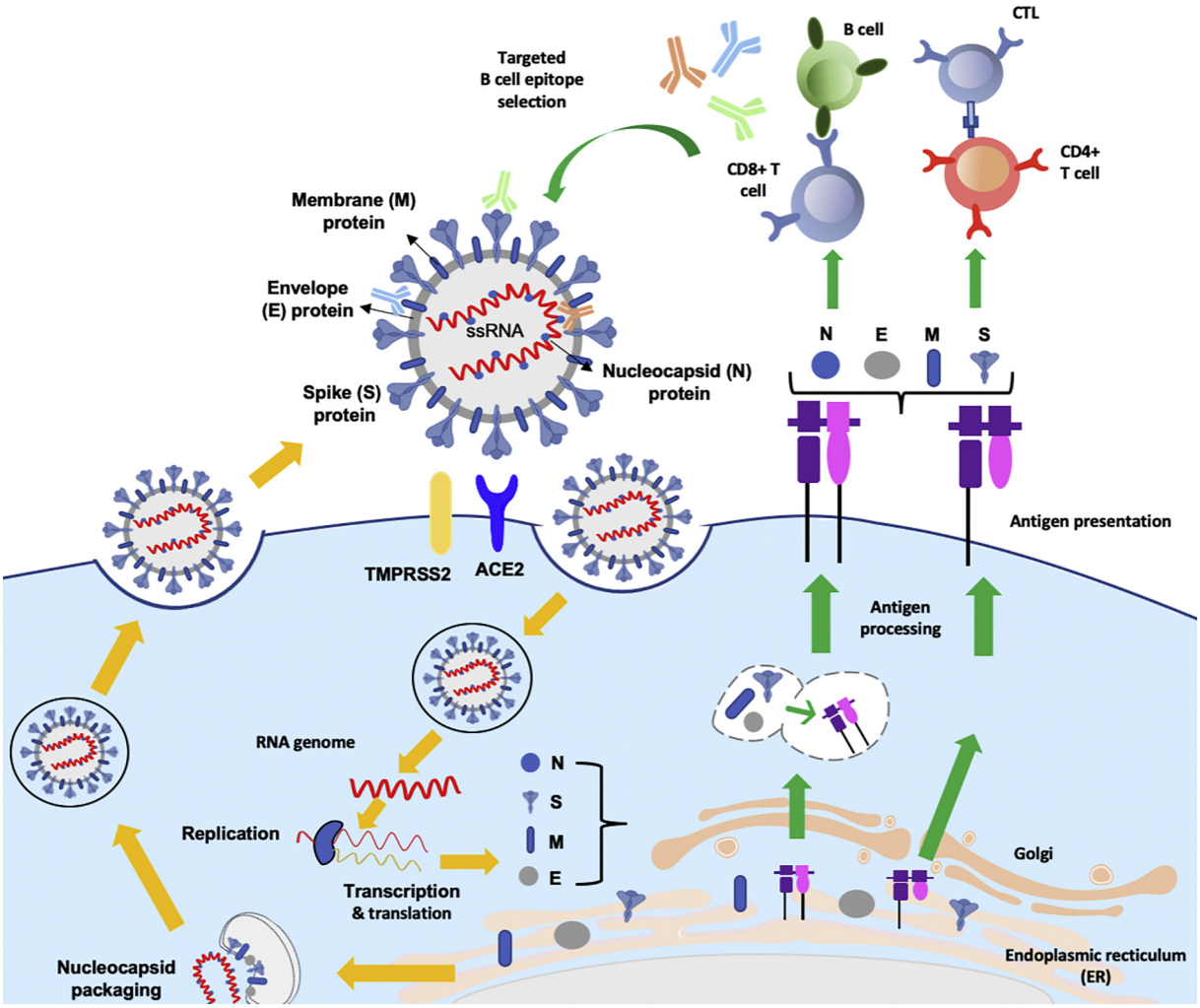

Fig. 5. SARS-COV-2 structure, infection, and cellular processing. SARS-CoV-2 binds to ACE2 and TMPRSS on the epithelial cell surface via the S protein which mediates virus-cell membrane fusion and entry. Viral genomic RNA is released and translated into viral polymerase proteins. Viral RNA is replicated and reverse transcribed in the cytoplasm, and the transcribed and translated viral proteins are transported into the endoplasmic reticulum (ER) and the Golgi. The S, E, and M protein are assembled in the ER-Golgi compartment and form a mature virion. The N protein assembles with mature virion, and a whole virus is released. The S, E, M, and N proteins are degraded into peptides and the viral antigens are presented by MHC I and II to $CD8^+$ T and CD 4^+ T cells, respectively, to induce cellular and humoral immunity.

stabilized S protein generated more neutralizing antibodies than the native S trimer has led to multiple SARS-CoV-2 vaccine candidates [224,225]. The RBD subunit has also been explored as a promising epitope to produce S antigen-specific neutralizing antibodies [113]. Among different S protein subunits including S1, RBD subunit, S2, and modified variants, the RBD subunit generated neutralizing antibodies at the highest tier in rabbits [226]. In addition, RBD-modified SARS-CoV-2 elicited a strong antibody response in rats [227].

5.3.1.2. T cell activation. Thus far, antibody generation by B cells has been the priority of most COVID-19 vaccine designs, with little focus on the clearance of SARS-CoV-2-infected cells by T cell-mediated cellular immunity and orchestration of other immune cells. $CD4^+$ T and $CD8^+$ T cells specific to the SARS-CoV-2 S, N, and M proteins can be found both in the patients who have recovered from COVID-19 and asymptomatic individuals [207]. A recent study to identify the correlation between neutralizing antibodies and $CD4^+$ T cells against the S protein [208] suggested vital roles of $CD4^+$ T cell activation in collaborative T and B cell activities against SARS-CoV-2. Antibody response to the N protein was developed in SARS-CoV patients [228] and its injection also elicited T cell responses in BALB/c mice [229]. However, targeting the N protein resulted in no protection from SARS-CoV, suggesting that the N protein could be a peripheral target in enhancing T cell immunity. Similar to SARS-CoV and MERS-CoV, SARS-CoV-2 prevents the development of antigen-specific T cells in order to evade immune response [230]. Both SARS-CoV and MERS-CoV replicate within double membrane vesicles to shield their viral RNA from degrading enzymes [231] and MERS-CoV downregulates genes related to antigen presentation [232]. While the mechanisms and results behind antigen presentation during SARS-CoV-2 infection remain unclear [41], it is still important to consider tackling these immunological pathways for multi-layered protection. Overall, COVID-19 vaccines should be able to deliver the SARS-CoV-2 S protein or peptide antigen for T cell stimulation, while minimizing the delivery of other fragments that have not demonstrated assistance in generating protective immunity.

5.3.2. Administration routes

Traditionally, vaccines are intramuscularly administered for sustained antigen release over time and greater likelihood of uptake and processing by APCs. However, growing evidence suggests that resident memory T cells remain present in the lung tissue and airways after the eradication of respiratory viral infections [112,233–235]. In a mouse influenza infection model, adoptive transfer of lung memory T cells demonstrated complete disease eradication within 8 days post-

infection, while animals that received adoptive transfer of the same memory T cells to the spleen succumbed to the disease [236]. In other studies, nasal or intramuscular vaccination with influenza antigen [237] or RSV DNA [238,239] both generated antigen-specific memory $CD8^+$ T cells in mice, but only nasal vaccination generated tissue-resident memory T cells that were capable of protecting the animal from a secondary exposure. For effective COVID-19 vaccination, lung-resident memory T cells should be primed against SARS-CoV-2 as they may be more effective than central and effector memory T cells [240]. Therefore, intranasal or aerosol-based inhalation could be a beneficial administration route to yield long-lasting immunity against SARS-CoV-2 [241]. This approach is being explored in the intranasal delivery of an adenovirus-based COVID-19 vaccine modified from the same platform for influenza vaccination [242]. Similar delivery routes can be easily applied to viral and nanoparticle vaccines but challenging for the delivery of cell-based vaccines.

Cost and accessibility are additional indispensable considerations in determining administration routes for COVID-19 vaccines. For example, intramuscular vaccinations are typically given by professional healthcare service providers in the United States, but trained medical personnel are not as available in many countries. Access to vaccines heavily depends on their formulations which are often delicate and require storage in coolers or cryotanks, presenting challenges in transportation and cold-chain distribution [243]. An oral adenovirus based-vaccine for SARS-CoV-2 S protein has been developed in response to these challenges, offering advantages of utilizing mucosal immunity with ease of storage and needle-free applications [244].

5.4. COVID-19 vaccine entities

Previous studies on SARS-CoV and MERS-CoV assisted in screening promising antigens to induce neutralizing antibodies against SARS-CoV-2 [114]. For example, SARS and MERS vaccines using the S1, RBD, and S2 subunits, showed high neutralizing antibody titers [245]. Once a desired antigen is identified, adjuvants, manufacturing process, and delivery methods need to be chosen for efficient and safe vaccination. In addition to the fast availability of the genome and structural information of SARS-CoV-2 [246], advanced vaccine platform technology is capable of contributing to rapid vaccine development and clinical translation [32]. Alternative to conventional antigen administration, nanotechnology platforms accommodate a wide variety of antigenic moieties for vaccine delivery, attributed to their flexibility for physical or chemical modifications [247]. Nano-sized materials from natural or synthetic sources can be engineered for desired size, administration route, and targeted delivery to immune cells (e.g., APCs) to achieve both adaptive and innate immune responses [248]. COVID-19 vaccines are being developed in the form of SARS-CoV-2 proteins and subunits, nucleic acids encoding a viral antigen, live-attenuated and inactivated viruses, replicating and non-replicating viral vectors, virus-like particles, and cell-based vaccines (Table 2). The immunological properties of the represented COVID-19 vaccine formulations and delivery platforms are listed in Table 3.

5.4.1. Protein subunits

Epitopes of the S, M, N and E proteins have been screened to identify the immune response for enhanced antibody production and T cell responses against SARS-CoV-2 [249,250]. Antigenicity along with structure and function of the S protein has been evaluated and the S2 subunit domain generated neutralizing antibodies against both SARS-CoV and SARS-CoV-2. Earlier vaccine studies on SARS-CoV and MERS-CoV patients using the full-length S proteins showed the presence of non-neutralizing antibodies contributing to ADE and increased systemic inflammation [251–253]. To avoid the generation of non-neutralizing antibodies, alternative subunit protein vaccines using RBD, S1, and S2 domains have been explored [113]. S2 protein subunit vaccines primed receptor binding and entry with antiviral effects. Targeting S1 protein and RBD prevented the entry to host cells thereby controlling viral infection [227]. Protein subunits have limited immunogenicity and require adjuvants in order to produce neutralizing antibodies. Whether targeting the full-length S protein or its subunits, both enhanced humoral and cellular immunity, including the generation of neutralizing antibodies [254]. Furthermore, the N protein has also been considered a promising vaccine material as it is highly immunogenic and abundantly expressed in SARS-CoV-2 [255]. T cell response against the S, M, and N subunit proteins has shown to be the most dominant and long-lasting in SARS-CoV-2 [207], and their subunit vaccines are popularly investigated in clinical trials [256]. Trimeric SARS-CoV-2 S protein subunit vaccines can be rapidly produced by expression in mammalian cells and resemble the native trimeric viral S protein [257], and another subunit particle of the S protein using the molecular clamp technology is undergoing clinical trials [258] (NCT04495933). Potent antigen-specific antibody responses were elicited within 2 weeks of immunization by microneedle patch to deliver S1 subunits [259].

5.4.2. Nucleic acids

Since the ability to elicit an immune response after injection of naked plasmid DNA was discovered, many clinical trials have used nucleic acids for vaccination against diverse diseases including infectious diseases and cancer [260,261]. Nucleic acids are attractive vaccine candidates as they are relatively simple to produce, safe to administer, and capable of generating high immunogenicity [262,263].

DNA vaccines are readily manufactured in large quantities, typically composed of a plasmid vector encoding a target vaccine molecule, and are capable of stimulation for long-term humoral and cellular immunity [264]. DNA is also stable and does not require cold storage, unlike conventional protein/peptide or whole virus vaccines [265]. Given the ubiquitous presence of RNase enzymes and structural differences, the half-life of DNA is longer as compared to mRNA, but DNA still needs to

Table 2
Compared COVID-19 vaccine forms.

Vaccine form	Antigen	Production	Advantages	Disadvantages
Inactivated virus	Whole virion	Virus particles inactivated by heat, chemicals or by radiation	Easy to prepare, safe, high-titer neutralizing antibodies	Possible cause of hypersensitivity
Live-attenuated virus	Whole virion	Attenuated the virulence keeping it viable	Rapid development, less adverse effects, induce high immune response	Phenotypic or genotypic reversion, not suitable to all age groups (safety testing)
RNA	S protein	Genetically engineered RNA for directly producing an antigen	Easy to design, higher degree of adaptability, induce strong immune response	Highly unstable, safety issues
DNA	S protein	Genetically engineered DNA for directly producing an antigen	Easy to design and scale up, high safety, high-titer neutralizing antibodies	Specific delivery tool required, Lower immune responses, repeated doses may cause toxicity
Recombinant protein	S protein	Antigenic components produced as a whole or subunit	High safety, consistent production, can induce cellular and humoral immune response	High cost, lower immunogenicity, require repeated dose and adjuvants
Viral vector-based vaccine	S protein	Genetically engineered with encoded target gene	Safety, induces high cellular and humoral immune responses	Possibility of presenting different immune responses

Table 3
COVID-19 vaccines in development.

Vaccine form	Developer	Platform	Route (duration, day)	Immunogenicity	Stage of development
RNA	mRNA-1273, Moderna/NIAID	Prefusion stabilized S protein mRNA encapsulated in LNP	IM (0, 28)	Repeated doses induce neutralizing antibodies and $CD4^+$ and $CD8^+$ T cell responses	Emergency use authorization by the FDA
	BNT-162, BioNTech/ Fosun Pharma/ Pfizer	3 LNP-formulation encapsulated mRNA	IM (0, 28)	High neutralizing and antibody titers and $CD4^+$ and $CD8^+$ T cells responses	Emergency use authorization by the FDA
	LNP-nCoVsaRNA, Imperial College London	Self-amplifying RNA packaged into tiny droplets of fat	IM	Published pre-clinical data show induction of neutralizing antibodies and T cells responses	Phase 1 ISRCTN17072692
	LUNAR-COV19, Arcturus/Duke-NUS	Lipid mediated delivery system for self-amlyfying mRNA	IM	Information suggests induction of high neutralizing antibody after single injection	Phase 1/2 NCT04480957
	CVnCOV/ Curevac	Lipid nanoparticle encapsulated mRNA	IM (0, 28)	High neutralizing antibodies and $CD4^+$ T cell responses	Phase 2 NCT04515147
	ARCoV/ People's Liberation Army (PLA) Academy of Military Sciences/ Walvax Biotech	mRNA expressing S protein	IM, (0, 14 or 0, 28)	Information suggests induction of neutralizing antibody in non-human primates	Phase 1 ChiCTR2000034112 ChiCTR2000039212
DNA	INO-4800, Inovio Pharmaceuticals	DNA plasmid vaccine with electroporation	ID (0, 28)	Safety and high overall immune response	Phase 1/2 NCT04447781 NCT04336410
	COVID-19 Vaccine/ Takara Bio, Osaka University	DNA plasmid expressing S protein + adjuvant	IM (0, 14)	N/A	Phase 1/2 NCT04463472 NCT04527081
	ZyCoV-D/ Zydus Cadila	DNA plasmid expressing S protein	ID (0, 28, 56)	Information shows immune responses in several animal species	Phase 1/2 CTRI/2020/07/026352
	GX19/ Genexine	DNA vaccine expressing S protein	IM (0, 28)	N/A	Phase 1/2 NCT04445389
Non-replicating viral vector	AZD1222 (ChAdOx1 nCoV-19)/ University of Oxford/ AstraZeneca	Chimpanzee adenovirus vector displaying Spike protein on its surface	IM	Strong immune response and high neutralizing antibodies with single injection (Low pre-existing immunity	Phase 3 ISRCTN89951424 NCT04516746 NCT04540393 CTRI/2020/08/027170
	Ad26 Cov S1, Janssen Pharmaceutical	Adenovirus serotype 26 expressing Spike protein	IM (0, 56)	Weak, requires boost injection	Phase 3 NCT04505722
	Gam-COVID-Vac Lyo/ Gamaleya Research Institute	rAd26 + rAd5 expressing Spike protein	IM (0, 21)	High neutralizing and antibody titers and CD4+ and CD8+ T cells responses using both lyophilized and frozen formulation	Phase 3 NCT04530396
	GRAd-S ReiThera/ LEUKOCARE	Replicative defective Simian Adenovirus (GRAd) encoding S	IM	N/A	Phase 1 NCT04528641
	Institute of Biotechnology, Academy of Military Medical Sciences, PLA of China	Non-replicating viral vector Ad5	IM/mucosal (0, 28)	N/A	Phase 1 NCT04552366
Replicating virus	Institute Pasteur/Themis/Univ. of Pittsburgh	Measles vector based	IM (0, 28)		Phase 1 NCT04497298
	Xiamen University	Intranasal flu based RBD	IM		Phase 1 ChiCTR2000037782
Inactivated virus	PiCoVacc/ Sinovac	Formalin inactivating whole virus particles + alum adjuvant	IM, (0,14 or 0,28)	Strong induction of neutralizing antibodies and enhanced immunogenicity	Phase 3 NCT04456595 669/UN6. KEP/EC/2020
	BBIBP-CorV Sinopharm/ Wuhan Institute of Biological Products/ Beijing Institute of Biological Products	Inactivated SARS-CoV-2	IM, (0, 14 or 0, 28)	Safe and high antibody titers	Phase 3 ChiCTR 2000034780
	Institute of Medical Biology, Chinese Academy of Science	Inactivated SARs-CoV-2	IM (0, 28)	N/A	Phase 1/2 NCT04470609
	Research Institute for Biological Safety Problems, Rep of Kazakhstan	Inactivated SARs-CoV-2	IM, (0, 21)	N/A	Phase 1/2 NCT04530357
	Bharat Biotech	Whole virion inactivated	IM (0, 14)		Phase 1/2 NCT04471519
Protein subunit	Novavax	Full length recombinant SARS-CoV-2 glycoprotein nanoparticles adjuvanted with Matrix M	IM (0, 21)	Unpublished pre-clinical data indicates high levels of S-specific neutralizing antibodies	Phase 2b NCT04533399
	Anhui Zhifei Longcom Biopharmaceuticals/ Institute of Microbiology, Chinese Academy of Sciences	Adjuvanted recombinant protein (RBD-dimer)	IM (0, 28 or 0,28,56)	N/A	Phase 2 NCT04466085
	Kentucky Bioprocessing Inc	RBD based	IM, (0, 21)	N/A	Phase 1/2 NCT04473690
	Sanofi Pasteur/ GSK	S protein (baculovirus production)	IM,	N/A	Phase 1/2

(continued on next page)

Table 3 (*continued*)

Vaccine form	Developer	Platform	Route (duration, day)	Immunogenicity	Stage of development
			(0, 21)		NCT04473690
	SCB-2019/ Clover Biopharmaceuticals Inc	Native like Trimeric subunit spike protein vaccine	IM, (0, 21)	Information suggest induction of neutralizing antibodies in animal models	Phase 1/2 NCT04530357
	Vaxine/ Medytox	Recombinant spike protein with Advax adjuvant	IM	N/A	Phase 1/2 NCT04530357
	University of Queensland	Molecular clamp stabilized Spike protein with MF59 adjuvant	IM (0, 28)	Pre-clinical results suggest high levels of neutralizing antibody	Phase 1 ACTRN12 +20000674392p ISCTN51232965
	Medigen Vaccine Corporation/ NIAID/ Dynavax	S-2P protein + CpG 1018	IM, (0, 28)	N/A	Phase 1 NCT04487210
	Instituto Finlay de Vacunas, Cuba	RBD+Adjuvant	IM, (0, 21)	N/A	Phase 1 IFV/COR/04
	FBRI SRC VB VECTOR, Rospotrednazor, Koltsovo	Peptide based protein subunit	IM, (0, 28)	N/A	Phase 1 NCT04527575
	West China Hospital, Sichuan University	RBD (baculovirus production expressed in sf9cells)	IM, (0, 28)	N/A	Phase 1 ChiCTR2000037518
	University Hospital Tuebingen	SARS-CoV-2 HLA DR peptides	SC	N/A	Phase 1 NCT04545749
	Ad5-nCoV, CanSino Biological Inc	Adenovirus serotype 5 expressing Spike protein	IM	Strong immune response with single delivery but impeded due to pre-existing immunity	Phase 3 NCT04526990 NCT04540419
	Ad26 Cov S1, Janssen Pharmaceutical	Adenovirus serotype 26 expressing Spike protein	IM (0, 56)	Weak, requires boost injection	Phase 3 NCT04505722 NCT04614948
	Gam-COVID-Vac Lyo/ Gamaleya Research Institute	rAd26 + rAd5 expressing Spike protein	IM (0, 21)	High neutralizing and antibody titers and CD4+ and CD8+ T cells responses using both lyophilized and frozen formulation	Phase 3 NCT04530396
	GRAd-S ReiThera/ LEUKOCARE	Replicative defective Simian Adenovirus (GRAd) encoding S	IM	N/A	Phase 1 NCT04528641
	Institute of Biotechnology, Academy of Military Medical Sciences, PLA of China	Non-replicating viral vector Ad5	IM/mucosal (0, 28)	N/A	Phase 1 NCT04552366
Replicating virus	Institute Pasteur/Themis/Univ. of Pittsburgh	Measles vector based	IM (0, 28)	N/A	Phase 1 NCT04497298
	Xiamen University	Intranasal flu based RBD	IM	N/A	Phase 1 ChiCTR2000037782
Inactivated virus	PiCoVacc/ Sinovac	Formalin inactivating whole virus particles + alum adjuvant	IM, (0,14 or 0,28)	Strong induction of neutralizing antibodies and enhanced immunogenicity	Phase 3 NCT04456595 669/UN6. KEP/EC/2020 NCT04582344 NCT04617483
	BBIBP-CorV Sinopharm/ Wuhan Institute of Biological Products/ Beijing Institute of Biological Products	Inactivated SARS-CoV-2	IM, (0, 14 or 0, 28)	Safe and high antibody titers	Phase 3 ChiCTR2000034780 ChiCTR2000039000 NCT04612972
	Institute of Medical Biology, Chinese Academy of Science	Inactivated SARS-CoV-2	IM (0, 28)	N/A	Phase 1/2 NCT04470609
	Anhui Zhifei Longcom Biopharmaceuticals/ Institute of Microbiology, Chinese Academy of Sciences	Adjuvanted recombinant protein (RBD-dimer)	IM (0, 28 or 0, 28, 56)	N/A	Phase 2 NCT04466085
	Kentucky Bioprocessing Inc	RBD based	IM, (0, 21)	N/A	Phase 1/2 NCT04473690
	Sanofi Pasteur/ GSK	S protein (baculovirus production)	IM, (0, 21)	N/A	Phase 1/2 NCT04473690
	SCB-2019/ Clover Biopharmaceuticals Inc	Native like Trimeric subunit spike protein vaccine	IM, (0, 21)	Information suggest induction of neutralizing antibodies in animal models	Phase 1/2 NCT04530357
	Vaxine/ Medytox	Recombinant spike protein with Advax adjuvant	IM	N/A	Phase 1/2 NCT04530357
	University of Queensland	Molecular clamp stabilized Spike protein with MF59 adjuvant	IM (0, 28)	Pre-clinical results suggest high levels of neutralizing antibody	Phase 1 ACTRN12 +20000674392p ISCTN51232965
	Medigen Vaccine Corporation/ NIAID/ Dynavax	S-2P protein + CpG 1018	IM, (0, 28)	N/A	Phase 1 NCT04487210
	Instituto Finlay de Vacunas, Cuba	RBD+Adjuvant	IM, (0, 21)	N/A	Phase 1 IFV/COR/04
	FBRI SRC VB VECTOR, Rospotrednazor, Koltsovo	Peptide based protein subunit	IM, (0, 28)	N/A	Phase 1 NCT04527575
	West China Hospital, Sichuan University	RBD (baculovirus production expressed in sf9cells)	IM, (0, 28)	N/A	Phase 1 ChiCTR2000037518

Table 3 (*continued*)

Vaccine form	Developer	Platform	Route (duration, day)	Immunogenicity	Stage of development
	University Hospital Tuebingen	SARS-CoV-2 HLA DR peptides	SC	N/A	Phase 1 NCT04545749
	Research Institute for Biological Safety Problems, Rep of Kazakhstan	Inactivated SARS-CoV-2	IM, (0, 21)	N/A	Phase 1/2 NCT04530357
	Bharat Biotech	Whole virion inactivated	IM (0, 14)	N/A	Phase 1/2 CTRI/2020/07/026300 CTRI/2020/09/027674 CTRI/2020/11/028976
Protein subunit	Novavax	Full length recombinant SARS-CoV-2 glycoprotein nanoparticles adjuvanted with Matrix M	IM (0, 21)	Unpublished pre-clinical data indicates high levels of S-specific neutralizing antibodies	Phase 2b 2020-004123-16 NCT04533399
	Anhui Zhifei Longcom Biopharmaceuticals/ Institute of Microbiology, Chinese Academy of Sciences	Adjuvanted recombinant protein (RBD-dimer)	IM (0, 28 or 0,28,56)	N/A	Phase 2 NCT04466085
	Kentucky Bioprocessing Inc	RBD based	IM, (0, 21)	N/A	Phase 1/2 NCT04473690
	Sanofi Pasteur/ GSK	S protein (baculovirus production)	IM, (0, 21)	N/A	Phase 1/2 NCT04473690
	SCB-2019/ Clover Biopharmaceuticals Inc	Native like Trimeric subunit spike protein vaccine	IM, (0, 21)	Information suggest induction of neutralizing antibodies in animal models	Phase 1/2 NCT04530357
	Vaxine/ Medytox	Recombinant spike protein with Advax adjuvant	IM	N/A	Phase 1/2 NCT04530357
	University of Queensland	Molecular clamp stabilized Spike protein with MF59 adjuvant	IM (0, 28)	Pre-clinical results suggest high levels of neutralizing antibody	Phase 1 ACTRN12 +20000674392p ISCTN51232965
	Medigen Vaccine Corporation/ NIAID/ Dynavax	S-2P protein + CpG 1018	IM, (0, 28)	N/A	Phase 1 NCT04487210
	Instituto Finlay de Vacunas, Cuba	RBD+Adjuvant	IM, (0, 21)	N/A	Phase 1 IFV/COR/04
	FBRI SRC VB VECTOR, Rospotrednazor, Koltsovo	Peptide based protein subunit	IM, (0, 28)	N/A	Phase 1 NCT04527575
	West China Hospital, Sichuan University	RBD (baculovirus production expressed in sf9cells)	IM, (0, 28)	N/A	Phase 1 ChiCTR2000037518
	University Hospital Tuebingen	SARS-CoV-2 HLA DR peptides	SC	N/A	Phase 1 NCT04545749

enter the nucleus for transcription, which may result in integration and mutation in the host genome [266]. Plasmid DNA vaccines do not elicit an appropriate immune response, requiring the need for multiple vaccinations or use of an adjuvant that promotes an increased adaptive immune response [267]. Upon expression of the transgene, APCs present antigenic peptides on their MHC I and II to prime T cells for initiation of humoral and cellular immunity. Plasmid DNA vaccines expressing SARS-CoV-2 S protein generated neutralizing antibodies and antigen specific $CD8^+$ T cells in mice and guinea pigs [268] and two repeated injections successfully protected rhesus macaques from COVID-19 [269]. Additional DNA plasmid vaccines expressing S protein are also undergoing phase I/II clinical trials (CTRI/2020/07/026352 and NCT04445389), in addition to a linear DNA vaccine against the S protein which is co-administered with alum adjuvant (N2CT04463472, NCT04527081).

In contrast to earlier studies using DNA, recent studies have explored the use of S antigen-encoding mRNA for vaccination [270]. mRNA vaccines need to be delivered to the cytoplasm of the host cell for translation to the target antigenic proteins. mRNA vaccines are a promising alternative to conventional protein or whole virus vaccines, attributed to their safety, high potency for generating an immune response, and rapid and low-cost manufacturing [263]. However, instability and inefficient *in vivo* delivery are barriers that require improvement, and various modifications and delivery methods have aimed to increase half-life *in vivo* and antigen translation [271–273]. An additional hurdle shared by DNA and mRNA vaccines is the penetration to the target cells. Advancement in nucleic acid delivery has increased the potential for nucleic acid vaccines to enhance immunogenicity [263,274]. Diverse methods have been applied for successful penetration of the lipid membrane including physical methods [275,276], cationic peptide delivery [277], lipid, and polymer-based delivery [278,279]. Lipid nanoparticles (LNPs) have become one of the most favorable tools for mRNA delivery consisting of four main components: An ionizable cationic lipid, lipid-linked polyethylene glycol (PEG), cholesterol, and naturally occurring phospholipids. In a clinical trial, 45 adults between ages 18-50 that were vaccinated with prefusion stabilized S protein-encoding mRNA encapsulated in LNPs, followed by a booster vaccination after 4 weeks, and generated neutralizing antibodies against pseudotyped lentivirus reporter and wild type SARS-CoV-2 [270]. Additionally, SARS-CoV-2-specific $CD4^+$ T cells were detected at doses of 25 and 100 μg while $CD8^+$ T cells were detected in the 100 μg-dosed group. There were no serious adverse effects but fatigue, chills, headache, myalgia, and pain at injection site were reported by some patients [270]. Phase I/II clinical trials on similar vaccines comprised of a nucleoside mRNA encoding the RBD domain or full-length S protein (NCT04449276, ISRCTN17072692, NCT04480957, NCT04368728) generated high immunogenicity with strong $CD4^+$ T and $CD8^+$ T cell responses [280] and similar results were also observed when injected to mice and rhesus macaques [281]. The preliminary outcomes of phase III clinical studies of mRNA vaccines claimed efficacy in preventing COVID-19 infection by 95% [282] and 94.5% [283]. Recently, Pfizer and BioNTech's, BNT162b2 became the

first mRNA-based vaccine to be authorized for emergency use by the UK on December 2, 2020 [284], and in the USA on December 11, 2020 [285,286] and is already being administered to essential workers. Additional mRNA vaccine platforms have been developed at an unprecedented speed and validated for immunogenicity and efficacy [287,288]. One of the biggest drawbacks of Pfizer and BioNTech's COVID-19 vaccine is long-term storage, as the vaccine must be stored at -70°C, creating challenges in reliable distribution. Despite the hurdles related to cold chain distribution, the mRNA vaccines for COVID-19 can help mitigate the global health crisis and provide an optimistic future for the application of mRNA towards preventing infectious diseases.

5.4.3. Live-attenuated and whole inactivated viruses

Traditional immunization against viral infections relies on the use of the whole pathogen in a weakened or inactivated state through chemical or physical alterations, and has resulted in many clinically successful vaccinations as the most immunogenic vaccine formulations [289]. Although they are known to be effective, risk of possible mutations leading to virulence reversal and reactivation in immune-compromised individuals is a factor to consider [290]. Studies have shown that a codon change in the viral genome can reduce the virulence reversion, albeit not applicable to all viruses [291]. Coronaviruses contain non-structural proteins that are not required for replication and can be altered leading to attenuation *in vivo*. For example, deletion of E proteins has been used as a strategy to attenuate several zoonotic coronaviruses [290,292,293]. Synthetic genome code expansion (GCE) technology for site-specific incorporation of unnatural amino acids into proteins [294,295] produced highly reproductive and genetically stable viruses with the ability to induce a robust antiviral response against MERS-CoV, and has also been applied to SARS-CoV-2 viral fragments [296,297]. Several live-attenuated virus vaccines, including one based on a RSV platform [298], are undergoing pre-clinical studies.

Inactivated viruses have been successfully applied against polio, hepatitis A, and influenza [299]. They can be rapidly produced by propagating viruses in cell cultures such as Vero cells, followed by heat or chemical inactivation using formaldehyde or beta-propiolactone [137]. They are incapable of replication due to the destroyed RNA and are safer than live-attenuated viruses, while expressing viral epitopes that can induce antibody responses [300]. However, the replication-incapability lowers immunogenicity of inactivated viruses and requires multiple injections along with adjuvants for long-term immunity [301]. Currently, there are six vaccine candidates using inactivated viruses with an additional eight candidates undergoing pre-clinical studies [256]. Purified inactivated SARS-CoV-2 administered with alum adjuvant protected rhesus macaques from COVID-19 by reducing viral titers and increasing neutralizing antibodies against the S and N proteins [302]. Another genetically stable inactivated SARS-CoV-2 vaccine was successfully produced at a pilot scale and induced high levels of neutralizing antibody titers in diverse animal models after immunization on days 0 and 14 at 2 μg/dose [137]. Although inactivated viruses can produce neutralizing antibodies, long-term immunity and safety needs to be further confirmed, as previous studies with inactivated SARS-CoV and RSV vaccines reported lung eosinophilia [303,304].

5.4.4. Recombinant viral vectors

Recombinant viral vectors are designed to express antigens of target pathogens and many different types of viral vectors are available [305]. Vast knowledge about the manipulation and functions of recombinant viral vectors as immunogens indicates them as highly versatile vaccine platforms [306]. Viral vectors have been engineered to deliver one or more antigens of choice, and the ability to load a relatively large genome offers the promise of developing a large variety of vaccines [307]. Delivery of target antigen by recombinant viral vectors produces potent antigens, mimicking the natural infection to induce strong T cells responses without the need for an adjuvant [308]. Rapid production additionally makes recombinant viral vectors a popular platform for vaccine delivery [305]. However, several design aspects of recombinant viral vectors need to be considered, such as potential integration into the host genome or incidental replications [309]. Non-replicating viral vectors are based on adenovirus type 5 (Ad5) and most of these vaccines are express the S protein or RBD subunit of SARS-CoV-2 [310]. However, individuals may already have pre-existing anti-adenoviral antibodies, so an alternative vector, chimpanzee adenovirus vector (ChAd) with low human prevalence, has been employed as a vaccine platform [311,312].

Ad5 encoding SARS-CoV-2 S protein that was designed to produce neutralizing antibodies upon intramuscular injection generated toxicity at a high dose and a low dose was further evaluated in Phase II clinical trials [310,313]. Previous phase II clinical trials of the same viral platform for an Ebola vaccine showed high initial levels of neutralizing antibodies but with rapidly declining antibody titer [314]. The majority of the volunteers in the Ebola vaccine study showed pre-existing immunity against Ad5, reducing the immunogenicity of the vaccine [315]. Ad26 encoding S protein demonstrated robust neutralizing antibody titers as its pre-existing immunity was lower than that of Ad5 [225]. This vaccine system has shown to be effective for HIV and Ebola vaccination in non-human primates and humans, respectively [316,317].

ChAd expressing S protein is one of the most clinically advanced COVID-19 vaccines. Individuals have low pre-existing immunity to the ChAd viral backbone [317] and previous studies on MERS [318] and the tuberculosis vaccine [319] confirmed the generation of high-titered neutralizing antibodies. In SARS-CoV-2 vaccination, high levels of neutralizing antibodies [320] were detected after a single vaccination, in addition to T cell responses [320]. Additional COVID-19 vaccines based on measles virus, parainfluenza virus 5, rabies virus, and adeno-associated virus (AAV) are being developed [256].

5.4.5. Virus-like particles

Virus like particles (VLPs) are produced by recombinant expression of structural proteins. Successful vaccines using this platform include the hepatitis B and human papillomavirus vaccines [321,322]. VLPs are highly stable, easy to scale up for production, and safe [323]. VLPs are structurally identical to the virus but lack the viral genome and are non-infectious. SARS-CoV-2 VLPs contain the S protein on the surface, allowing for particle fusion to host cells via the ACE2 receptor and priming using TMPRSS2 but unlike subunit vaccines, the VLPs are incapable of directly binding to B cell receptors for antibody production [324]. SARS-CoV-2 VLPs produced by expressing desired genes in mammalian cells maintained the structural and morphological properties of the virus, making VLPs a promising vaccine candidate and powerful tool for research in understanding the mechanism of SARS-CoV-2 [108]. SARS-CoV-2 VLPs produced from genetically engineered plants suggested efficacy in generation of neutralizing antibodies [325] and are undergoing phase I clinical trials (NCT04450004). VLPs comprised of hepatitis B surface antigen further engineered to carry the SARS-CoV-2 S protein on the surface are undergoing phase I clinical trials (ACTRN12620000817943).

5.4.6. Cell-based vaccines

Reprogramming the immune system against infectious diseases and cancers using engineered cells has shown clinical promise in recent years and DCs are a particularly interesting immunotherapeutic target, given their ability to uptake and present antigen through various mechanisms and prime potent effector responses [326]. Apart from direct antigen presentation, DCs are characterized to migrate between lymphoid and non-lymphoid issues and modulate cytokines to control inflammation for long-lasting immunological effects [327]. Cell-based therapy has been applied to COVID-19 vaccines. In one example, a "synthetic mini-gene" expressing the S, M, E, N, and polyprotein protease (P) SARS-CoV-2 viral proteins were engineered using a lentiviral vector (LV-SMENP) and delivered to artificial APCs (aAPCs) (NCT04276896). The primary outcome of the approach was to generate an immune response against SARS-CoV-2. In addition, LV-SMENP-DCs from the

same institute further modified DCs to express viral antigens which can activate cytotoxic T-cells *ex vivo* (NCT04299724). AV-COVID-19 from Aivita Biomedical Inc. uses autologous DCs loaded with antigens from SARS-CoV-2 and is undergoing phase I clinical trials (NCT04386252). However, cell-based vaccines are hampered by low productivity and high cost.

5.5. Vaccine production, formulation, and immune modulation

Currently, education of $CD4^+$ T, $CD8^+$ T, and B cells against SARS-CoV-2 S protein appears to be the most feasible and attainable strategy [207]. This is the protein which has proven to be the most unique to the virus, directly permits cell infection, will best account for viral mutation over time, and generates the most consistent immune response [113,114]. While there are many platforms being developed for vaccination, it is challenging to determine which will be most appropriate to meet all clinical demands and suitability for production and distribution in a timely manner.

5.5.1. Molecular and viral vaccines

Viruses of choice must first be propagated in cells, isolated, and purified. For the influenza vaccine, the propagation is accomplished in fertilized chicken eggs [328]. However, SARS-CoV-2 does not replicate in chicken eggs [329] and demands an alternative scalable production method which is typically costly to develop and often requires specialized equipment. Inactivated viral and molecular vaccines contain formaldehyde used in the deactivation process, aluminum salt as an adjuvant to boost the immune response, preservatives, stabilizers, and antibiotics [330]. While whole attenuated, inactivated, and fragmented SARS-CoV-2 present simplicity in design, they also contain all the viral proteins including non-essential antigens that waste valuable space on antigen presenting machinery and decrease the yield of potent antigens [331]. Therefore, whole viruses may not be the best option for vaccination.

Conventionally, antigen delivery is accomplished by administration of entire or fragmented pieces of disabled virus. While this approach has been extremely effective for vaccination against certain viruses such as influenza and polio [299], it has not demonstrated the same efficacy for other viruses, such as HIV [332] and closely related MERS [333]. Therefore, it is possible for this approach to be ineffective for COVID-19 vaccination. Additionally, while this approach is clinically proven to be safe, inactivated viral vaccines do not typically induce long-term immunity, as evidence by the need for several doses over time [331,334]. By vaccinating patients with the whole virus, APCs could uptake and break it down into multiple antigenic peptides for the greatest opportunity to generate an immune response, without the risk of infection [335]. Furthermore, viruses may be directly recognized by B cells for neutralizing antibody production. This vaccine platform also has the additional benefit of containing some functional RNAs, which have also proven to assist in generating immune response [335,337]. As these viruses are limited in activity, they may only be administered to individuals with healthy, acive immune systems, which excludes young children, older individuals, and anyone with underlying health conditions or undergoing immune-compromising treatments [338,339]. SARS-CoV-2 has been particularly dangerous for the latter two groups, making the exclusion unacceptable. SARS-CoV-2 production is challenging along with other risk factors, and alternative viral vaccine platforms are being explored. For example, recombinant adeno-associated virus (AAV), used in FDA-approved gene therapies, is proven to be safe and non-carcinogenic [340]. Antigen encoding AAV can safely delivery transgenes processed by DCs for T and B cell immune responses, and recombinant AAV expressing the RBD of SARS-CoV induced sufficient S protein neutralizing antibodies [341].

5.5.2. Nanoparticle vaccines

Diverse delivery systems have attracted considerable attention since they can be designed to protect and deliver antigen and adjuvant payloads, and assist in multivalent antigen presentation, contributing to more effective vaccinations with enhanced immune response [342]. Advances in nanotechnology offer great potential in vaccine design and have helped in development of novel candidate vaccine formulations at unprecedented speed [32]. Vaccine carriers capable of controlled release, such as polymer matrixes and hydrogels, or newly developed nanotechnology platforms with desirable physical, chemical, and biological properties are promising for the targeted delivery of antigens and the prevention of disease transmission.

Production of nanoparticle-based vaccines heavily depends on the materials (polymer, protein, lipids, and cellular vesicles) and the payload (peptide, protein, RNA, DNA, and whole virus) of choice. In this case, a scalable amount of SARS-CoV-2 vaccine payload will need to be readily available. This will entail the mass biological production of viruses and S protein, or synthesis of the S protein and/or peptides. Large-scale production of nucleic acid vaccine payloads may be more achievable than viruses and antigenic proteins, as these can be collected from small batches and amplified [343]. Like their molecular counterparts, nanoparticle vaccines often require co-administration or co-encapsulation of an adjuvant for efficient DC stimulation. Co-encapsulation ensures the adjuvant and the antigenic payload reach the target site at the same time, which is critical for DC antigen processing and maturation [344]. Nanoparticle vaccine formulation is advantageous over administrating molecular vaccines alone because the payload is protected from immature degradation and unintended targets such as macrophages. Furthermore, recent progress has allowed for the preparation of nanoparticles with desired physiochemical properties including size, shape, solubility, hydrophilicity, hydrophobicity, and surface modification, making them highly tunable for vaccine delivery [345,346]. Additionally, refrigerated storage is not necessary for lyophilized nanoparticles. Nanoparticle-based formulations may increase uptake by phagocytic APCs, particularly when targeting moieties are incorporated on their surface [347]. Co-delivery of adjuvant makes APC-targeting nanoparticles ideal vaccines for efficiently and properly educating T and B cells including high antibody production [344]. Chitosan and dextran sulfate-based polymeric nanoparticles, inorganic nanoparticles such as AuNPs, and carbon nanotubes have been exploited for vaccination [348]. Poly lactic-co-glycolic-acid (PLGA) nanoparticles are suitable for encapsulating antigens for controlled release and induced prolonged cellular and humoral immunity [349]. LNPs are promising carriers for mRNA vaccines due to their ability to efficiently deliver payloads into the cytoplasm of a target cell via parenteral administration and subsequently generate high-titered antibodies and enhanced T cell immunity [263].

5.5.3. Adjuvants

The aforementioned vaccines require co-delivery of adjuvants for proper immune stimulation and response [350]. Adjuvants are natural or synthetic materials that are readily recognized by the immune system and enhance the desired immune response [351]. In particular, aluminum salts have been utilized in vaccines for over 70 years. Adjuvants currently investigated in COVID-19 vaccine formulations are summarized in Table 4. Alum adjuvants in combination with COVID-19 S protein demonstrated increased neutralizing antibody production [352,353], but did not promote T cell activation. Newer adjuvants developed for rapidly and specifically activated immune responses [353], emulsions and toll-like receptor (TLR) agonists, have shown to induce both B and T cell responses. The full desired immune response can be achieved by combined use of multiple adjuvants. All adjuvants must be confirmed for safety and efficacy via clinical trials, making the use of an already approved adjuvant a realistic strategy for timely development of COVID-19 vaccines [350]. Many front runner COVID-19 vaccines under development are adjuvant-free formulations and are able to elicit a potent immune response on their own without an

Table 4
Adjuvants for COVID-19 vaccines

Adjuvant	Examples	Immune response in COVID-19 vaccines	References
Aluminum	Amorphous aluminum hydroxy phosphate sulfate (AAHS), aluminum hydroxide, aluminum phosphate, potassium aluminum phosphate (Alum)	B cell activation, high IgG1 titers, increased production of neutralizing antibodies, enhanced long lasting memory B cells, immunopathologic reactions	[212,353,354,356–358]
Emulsion	AS03, MF59	Generation of humoral and cellular immune responses, high titers of neutralizing antibodies, CD4+ and CD8+ T cell activation	[353,359–360]
TLR agonist	CpG	IgG production, IgA production, neutralizing antibodies, CD4+ T cell activation, CD8+ T cell activation, production of memory CD8+ T cells	[353,361–363]

adjuvant [282,283,354]. Adjuvant-free vaccine formulations can dramatically save on development and testing time as well as manufacturing cost. However, they may not reach the maximum vaccine potency and long-lasting immunity, and the majority of classical forms of COVID-19 vaccines such as inactivated or attenuated viruses, viral fragments, or antigenic proteins or peptides will likely require addition of adjuvants [350,355]. With the rapid evolution of COVID-19 vaccines, roles and necessities of adjuvants for desired immunity will be elucidated and result in optimal formulations.

5.5.4. APC vaccines

Patient-isolated DCs by leukapheresis are used for personalized cell therapy for cancer [327]. While clinical trials demonstrated safety and increase of survival of the patients, DC therapy has not come to fruition due to multiple challenges related to time-consuming and labor-intensive preparation, limited scalability, and difficulty controlling DC maturation state which dictates how and at what magnitude the immune system responds [326]. Despite "off the shelf" versions of DCs and allogenic and HLA-matching DCs which are readily available, DC therapy is still under exploration [364]. DCs need to be pulsed with proteins, peptides, or viruses for antigen presentation along with appropriate stimulations [365]. If a rapid and reliable production of SARS-CoV-2 antigenic peptides was available, DCs could be directly incubated with the peptides for size-restricted presentation by MHC I and II [366]. Immune cells are designed with inherent plasticity to respond to microenvironmental and temporal cues, which is beneficial for immune activation when they are at desired states. For example, DC-based COVID-19 vaccines may require pre-conditioning to a stable mature state and active migration to lymph nodes [326,367]. Furthermore, DC-based vaccine formulations are typically difficult to quality check, attributed to batch to batch variation, especially before and after freezing and thawing. [368]. The access to cell-based vaccines are limited due to high production cost, stringent storage and transport requirements, and limited availability [368]. Properly engineered DC-based COVID-19 vaccines could effectively stimulate B and T cells via actively migrating to lymph nodes where they directly present antigenic peptides of SARS-CoV-2 S protein on MHC I and II to effector immune cells along with stimulation with appropriate cytokines [366] (Fig. 6).

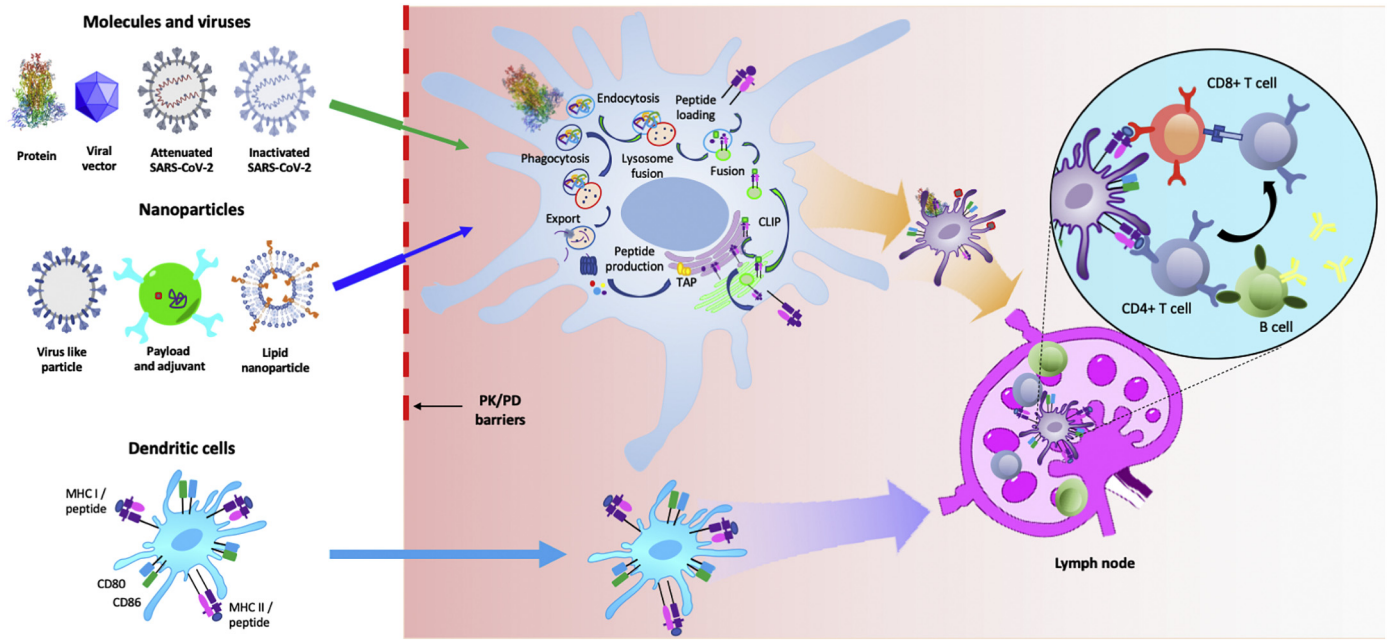

Fig. 6. Molecular and viral vaccines post-injection act as a depot site, necessitating uptake by local cells for antigen presentation. Vaccines from this category require recruitment of APCs (e.g., DCs) to the vaccination site for subsequent antigen processing and transportation to the lymph nodes. Importantly, these vaccine formulations typically include adjuvant for immune stimulation including desired DC maturation. Nanoparticle formulations also accompany adjuvant and ensure the co-delivery of both adjuvant and the vaccine payload to DCs, resulting in increased likelihood of DC maturation against the co-delivered antigens. Targeting moieties also assist nanoparticles in reaching the DC. DCs can also be isolated and engineered *ex vivo* to present SARS-CoV-2 antigens for the optimized antigen presentation in a desired maturation state. While this process is time-consuming, expensive, and difficult, direct deployment of antigen presenting DCs avoids pharmacokinetic (PK) and pharmacodynamic (PD) barriers, such as clearance associated with molecular and viral vaccines, and the cell recruitment steps required by other vaccination methods. Activated DCs migrate through the lymphatics to the lymph node where they can educate T and B cells for active clearance of infected cells and antibody production.

6. Perspectives

In order to protect the majority of global populations, including high-risk groups, more than 200 COVID-19 vaccine candidates are under investigation (Fig. 1) by adapting previously developed vaccine platforms and applying knowledge of infectious viruses including HIV, influenza, Zika, Ebola, SARS-CoV, and MERS-CoV [18,219,318]. Administration of SARS-CoV-2 proteins and peptide subunits and genetic materials encoding the viral antigens requires formulations that meet the clinical requirements for efficient and safe vaccination, such as high stability under physiological conditions, minimized off-target effects, and efficient cellular uptake by target immune cells [253,299]. The potential advantages of formulating COVID-19 vaccines in various nanotechnology platforms are increased stability of the vaccine materials for prolonged immunization, improved pharmacokinetics, and pharmacodynamics of the antigens, and enhanced immune response by co-delivered adjuvants. This is a particularly important design consideration for emerging forms of nucleic acid and cell-based COVID-19 vaccines as already demonstrated by the current clinical trials [26,32]. A significant challenge in COVID-19 vaccine development is frequent mutations in the SARS-CoV-2 S protein, the most common target antigen in the current effort, limiting the efficacy of the first generation COVID-19 vaccines and even requiring the recovered patients to be vaccinated against new mutants [35,119,222]. Therefore, an ideal COVID-19 vaccine platform must allow easy and rapid accommodation of newly mutated and identified antigens. The current ongoing pre-clinical and clinical studies on COVID-19 vaccines primarily target the generation of neutralizing antibodies against SARS-CoV-2 and desirably inducing production of memory T and B cells [114,206,208]. Efficient and long-term protection from SARS-CoV-2 infection requires a well-orchestrated innate, humoral, and cellular immunity, which can be achieved by a vaccine delivery platform that integrates multiple potent antigens or antigen-encoding nucleic acids, co-delivers appropriate co-stimulatory molecules, and targets specific immune cells.

Inflammation is an essential part of immune response and required to eliminate pathogens, followed by tissue regeneration and homeostasis restoration [369]. In contrast to other infections where inflammatory activity is beneficial, excessive production of cytokines/chemokines during SARS-CoV-2 infection, called the cytokine storm, leads to ARDS or multiple-organ dysfunction [99,103]. Controlling the activity of cytokines/chemokines in the early stages of COVID-19 using immunomodulators, cytokine antagonists, and reduction of inflammatory cell infiltration to the site of inflammation is reported to help reduce patient mortality rates [76,103,370]. As combined drug therapy offers lowered dosage, reducing the side effects, and the likelihood of developing drug resistance [371], COVID-19 vaccines using a nanotechnology platform are able to co-deliver antigens and additional desired drugs such as protease inhibitors, reverse transcriptase inhibitors (e.g., remdesivir), and immune suppressants (e.g., dexamethasone) to prevent a cytokine storm [372] or synergistically eradicate SARS-CoV-2 before severe disease progression (Fig. 7). Nanoparticles with cytokine-binding properties quenched the cytokine storm, and PEGylated dextran-coated super magnetic iron oxide nanoparticles (SPION) tethered with anti-IL4Rα antibodies decreased pro-inflammatory cytokine expression and repaired lung tissue in mice [373]. ACE2-expressing $CD68^{+}$ $CD169^{+}$ macrophages that contain the SARS-CoV-2 N protein increased IL-6 in spleen and lymph nodes [374], and using nanoparticles that target the inflammatory cells responsible for cytokine storm is a promising approach to developing efficient and safe COVID-19 vaccines. Recent studies showed biomimetic nanomaterials such as extracellular vesicles (EVs) carrying anti-inflammatory drugs to calm the local cytokine release syndrome in pneumonic lungs [375]. Furthermore, ACE2- and CD147-expressing cellular nanosponges were found to bind and inhibit SARS-CoV-2 infection [376].

While the extremely unusual circumstance requiring immediate production and availability of COVID-19 vaccines has led the leading candidates to advanced clinical stages at remarkable paces, robust clinical assessment on potential risks must be made and considered before vaccination to unprecedently large populations [32,58,240]. While still relatively rare, some patients in clinical trials experienced serious side effects that were not fully explained [377]. Re-opening of communities and countries based on the release of ineffective or partially effective vaccines could have dire impacts as also indicated by the FDA's requirements for rigorous trials before vaccine approval [378]. A premature statement could have unintended consequences, such as the unproven promise of hydroxychloroquine in COVID-19 treatment that led to a shortage for Lupus patients [379]. The efficacy and durability of immunity by COVID-19 vaccines are unknown and may require a two-dose heterogeneous prime-boost strategy or a periodic booster dose [380,381]. Pre-existing antibodies against SARS-CoV-2 or other viruses and their cross-reactivity may also play roles in efficacy and safety of COVID-19 vaccines [207] but having individuals checked before vaccination could be helpful but a daunting task.

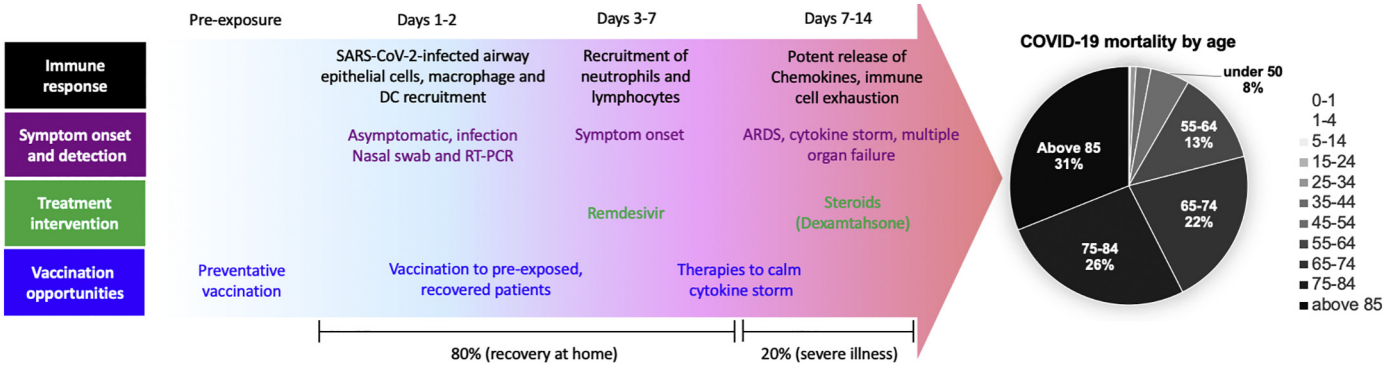

Fig. 7. Clinical disease development of COVID-19 and combined vaccination and treatment strategy for synergistic SARS-CoV-2 eradication, minimized side effects, and lowered mortality. Within 1-2 days post infection, SARS-CoV-2 locally replicates, and most individuals are asymptomatic. In the following 3-7, the virus continues to spread to the respiratory tract and the lungs with clinical manifestation. Inflammatory cells release high levels of cytokines (e.g., IL-6, IL-1β, and IFNα), contributing to a cytokine storm. In the later stages of infection, COVID-19 patients become severely ill, leading to ARDS, multiple organ failure, and eventually death. Most treatment interventions have focused on repurposed anti-viral or anti-inflammatory drugs and the FDA approved the use of remdesivir and dexamethasone for inhibition of viral RNA synthesis and to dampen inflammation, respectively. Controlling both virus replication and inflammation can help reduce patient mortality rate but their clinical efficacy is limited. Diverse nanotechnology platforms can be designed for combination therapy by the delivery of multivalent antigens and desired drugs which can synergistically reduce viral load, inflammation, and severe disease progression.

7. Conclusion

The unique, unexpected pathological and epidemiological natures of COVID-19 require unprecedented investments of efforts and resources, particularly in the development of effective and safe vaccines. The prior clinical experience with SARS-CoV and MERS-CoV is still limited to warrant timely, global availability of COVID-19 vaccines, which has been compensated for and overcome by global collaborations and competitions among pharmaceutical industry and the research community, starting with the historically fast clinical trials. In accomplishing high titered neutralizing antibodies and cellular immunity, potent antigens need to be formulated in vaccine carriers that are designed not only to deliver the payloads to target cells along with appropriate stimulations but also address specific challenges in COVID-19 vaccination such as long-term immunity and avoided cytokine storms. A broad library of nanomaterials originally developed for the delivery of peptides, proteins, nucleic acids, viruses, and cells are promising to meet the requirements for COVID-19 vaccines. COVID-19 is an enormous scientific, clinical, and societal challenge that innovative vaccine formulations play critical roles to overcome.

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