Review

Understanding the Molecular Actions of Spike Glycoprotein in SARS-CoV-2 and Issues of a Novel Therapeutic Strategy for the COVID-19 Vaccine

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Abstract: In vaccine development, many use the spike protein (S protein), which has multiple “spike-like” structures protruding from the spherical structure of the coronavirus, as an antigen. However, there are concerns about its effectiveness and toxicity. When S protein is used in a vaccine, its ability to attack viruses may be weak, and its effectiveness in eliciting immunity will only last for a short period of time. Moreover, it may cause “antibody-dependent immune enhancement”, which can enhance infections. In addition, the three-dimensional (3D) structure of epitopes is essential for functional analysis and structure-based vaccine design. Additionally, during viral infection, large amounts of extracellular vesicles (EVs) are secreted from infected cells, which function as a communication network between cells and coordinate the response to infection. Under conditions where SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) molecular vaccination produces overwhelming SARS-CoV-2 spike glycoprotein, a significant proportion of the overproduced intracellular spike glycoprotein is transported via EVs. Therefore, it will be important to understand the infection mechanisms of SARA-CoV-2 via EV-dependent and EV-independent uptake into cells and to model the infection processes based on 3D structural features at interaction sites.

Keywords: COVID-19; double-membrane vesicle; lipid nanoparticle; SARS-CoV-2; TMPRSS2

1. Introduction

As the novel coronavirus disease (COVID-19) spreads worldwide, the development of a vaccine for COVID-19 using AI (artificial intelligence) analysis is progressing, applying the technology and know-how from developing a cancer vaccine [1]. As diagnosis, treatment, and preventive measures for COVID-19 are needed as soon as possible, the movement of drug development is expanding beyond boundaries into the information and communications field. In the development of a new coronavirus vaccine, the focus is on which proteins of the new coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), to use as a vaccine. For a vaccine to be effective, it must be recognized by the recipient as an antigen and elicit an immune response that attacks this antigen [2]. In vaccine development, many use the spike protein (S protein), which has multiple “spike-like” structures protruding from the spherical structure of the coronavirus, as an antigen [3–5]. However, there are concerns about its effectiveness and toxicity. When S protein is used in vaccines, its ability to attack viruses may be weak, and its effectiveness in eliciting immunity will only last for a short period of time [6,7]. Moreover, it may cause side effects on the lungs or cause “antibody-dependent immune enhancement”, which can enhance infections [8]. In many vaccines’ development, while movements targeting S proteins are
becoming more active, vaccine development may end in failure if S proteins are the only target [9,10]. Therefore, attention is being paid to a completely new approach that expands the entire range of viral proteins to antigen candidates that can be used as vaccines, and AI technology will be the driving force behind this approach [1,11].

The main targets of vaccines in living organisms are T cells, which eliminate abnormal cells, and B cells, which produce antibodies that attack abnormal cells. Antigens are taken up by antigen-presenting cells, which convey the presence of antigens to T cells and B cells, but there are various human leukocyte antigens (HLA), and there are differences in immune responses. Based on these mechanisms, it is important to create vaccines that are more reliably recognized as antigens and more reliably activate immune cells [12]. An epitope is a site that becomes a target for immune attack. In addition to forming part of the proteins that protrude on the surface of the virus, such as the spike protein, it is present in various proteins of the virus. Among these, it is possible to identify a promising “hot spot” using AI analysis. By comparing data on different types of viruses and excluding areas with frequent genetic mutations, it is possible to avoid the ineffectiveness of vaccines due to genetic mutations of the virus [13,14]. As the virus repeatedly infects, mutants appear, in which the amino acid at the site recognized by the antibody is accidentally replaced with another amino acid due to mutations. If this mutant virus is not recognized as pathogenic by antibodies produced during a previous infection, individuals who have been infected once can also become infected [15–17]. These amino acid changes that allow the virus to escape from binding with antibodies are called antigenic mutations [18–20]. This viral genetic information is used to elucidate virus transmission routes between different host animals and to estimate antigen-determining sites in past antigenic mutations [21]. Attempts to predict future antigenic variations based on viral genetic information will lead to the development of useful vaccines [22]. Furthermore, based on key HLA data, vaccination can cover as wide a population as possible. After such analysis, the designed vaccine features can combine multiple epitopes into one vaccine [23–27]. Rather than making a vaccine from a single antigen, as exemplified by the S protein, in addition to collecting multiple narrowed-down epitopes, it is possible to select the best part of the entire genome of all viruses to select epitopes that lead to immune activation [28,29].

The challenges and limitations of previous work on COVID-19 vaccines have been multifaceted. Some key factors include the following:

1. Maintaining research and development incentives: one challenge has been to sustain strong research and development incentives to drive vaccine innovation [30].
2. Financial investment and demand: while unprecedented financial investments and massive demand have accelerated vaccine development, they also pose challenges in terms of sustainability and equitable distribution [31].
3. Scientific innovations and regulatory reviews: previous scientific innovations, accelerated clinical development, and regulatory reviews have been crucial but also come with challenges in ensuring safety and efficacy standards are met.
4. Limitations in vaccination promotion: challenges exist in promoting vaccination due to factors like limitations in the target age population and breakthrough infections [32].

These challenges highlight the complex landscape of COVID-19 vaccine development, emphasizing the need for ongoing research, collaboration, and innovation to address them effectively.

The development of COVID-19 vaccines has been a critical aspect of combating the pandemic. The following are some key points regarding the issues and complications related to the development of COVID-19 vaccines:

I. Rapid development: COVID-19 vaccines have been developed at an accelerated pace compared to traditional vaccines, with 232 vaccine candidates, 172 in preclinical development, and 60 in clinical development. This rapid development has aimed to significantly reduce the typical 10- to 15-year timeline to 12 to 24 months [33,34].

II. Vaccine types: Various types of COVID-19 vaccines have been developed, including mRNA vaccines like BNT162 by Pfizer/BioNTech and mRNA-1273 by Moderna,
adenovirus vector vaccines like AstraZeneca and Jenssen, and inactivated killed vaccines like Sinopharm. These vaccines have shown high efficacy in preventing severe illness and death [35].

(III) Challenges: The accelerated process of COVID-19 vaccine development and Emergency Use Authorization (EUA) have raised unanswered questions. Additionally, the emergence of new strains of SARS-CoV-2 has posed challenges for vaccine developers and governments worldwide [33].

(IV) Safety concerns: Studies have shown that COVID-19 vaccines are generally safe, with lower rates of death among vaccinated individuals compared to those who are not vaccinated. Adverse events post-vaccination are generally mild to moderate, with severe reactions being rare. Adverse events associated with COVID-19 vaccines include rare effects like anaphylaxis, blood clots, myocarditis, pericarditis, hearing changes, and tinnitus. However, the overall risk of severe adverse effects is low, and healthcare professionals monitor and manage any reactions carefully [36].

(V) Vaccine efficacy: Studies have shown that COVID-19 vaccines significantly reduce the risk of death from COVID-19 and its complications. Vaccinated individuals are not at a greater risk of death from non-COVID causes compared to unvaccinated individuals.

(VI) Long-term effects: While concerns about long-term side effects exist, current data suggest that severe effects following vaccination are rare.

While the development of COVID-19 vaccines has been a remarkable achievement (Table 1), challenges such as rapid development timelines, addressing new variants, ensuring safety, and managing distribution remain crucial aspects in the ongoing fight against the pandemic [35].

Table 1. Vaccine design for COVID-19.

<table>
<thead>
<tr>
<th>Pharmaceutical Company</th>
<th>Products</th>
<th>Platform</th>
<th>Design</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pfizer-BioNTech (Mainz, Germany)</td>
<td>BNT162b2</td>
<td>mRNA</td>
<td>Full-length S protein</td>
</tr>
<tr>
<td>Moderna (Cambridge, MA, USA)</td>
<td>mRNA-1273</td>
<td>mRNA</td>
<td>Two proline substitutions (K986P and V987P)</td>
</tr>
<tr>
<td>Novavax (Gaithersburg, MD, USA)</td>
<td>NVX-CoV2373</td>
<td>Protein subunit</td>
<td>Full-length S protein</td>
</tr>
<tr>
<td>Janssen (New Brunswick, NJ, USA)</td>
<td>INN-78436735</td>
<td>Adenovirus</td>
<td>Full-length S protein</td>
</tr>
<tr>
<td>Oxford-AstraZeneca (Cambridge, UK)</td>
<td>AZD1222</td>
<td>Adenovirus</td>
<td>Full-length S protein</td>
</tr>
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When comparing Moderna and Novavax COVID-19 vaccines, several key differences emerge. Moderna is an mRNA vaccine, while Novavax is a protein subunit vaccine. In terms of effectiveness, both vaccines help protect against severe illness, hospitalization, or death caused by COVID-19. Moderna has shown a strong immune response against certain variants, with antibody responses significantly higher against specific variants compared to Novavax. Novavax had a 90% efficacy in its clinical trial and performed almost as well as mRNA vaccines in early trials. Regarding dosing, Moderna's primary series consists of two doses for individuals aged six months and older, while Novavax's dosing varies based on age groups and vaccination history. Side effects for both vaccines include common reactions like pain at the injection site, tiredness, headache, and fever, with rare cases of serious allergic reactions reported for both vaccines. In terms of ingredients, Moderna and Novavax differ significantly. Moderna contains mRNA, fats to help the mRNA, sugar for
stability, and acidic preservatives or latex in the viral stoppers. On the other hand, Novavax includes proteins to induce an immune response, fats to assist the protein, salts for stability, and sugar for stability as well.

The comparison between the Oxford-AstraZeneca and Pfizer-BioNTech COVID-19 vaccines reveals some key differences. The Pfizer-BioNTech vaccine has shown a higher efficacy in preventing symptomatic infections, with an efficacy rate of 95% compared to AstraZeneca’s 70.4% efficacy. Additionally, research indicates that the Pfizer-BioNTech vaccine may be associated with a relatively low reduction in the rate of SARS-CoV-2 after a single dose, while the Oxford-AstraZeneca vaccine has shown a marked reduction in outcomes related to severe COVID-19 [37]. Furthermore, the effectiveness of a single dose of either vaccine is more pronounced in older individuals, with no significant difference observed between the two vaccines at 3-month follow-up [37].

The comparison between the JNJ-78436735 (Johnson & Johnson) COVID-19 vaccine and the Moderna vaccine also reveals some key differences. According to the data, the odds ratio of mRNA-1273 (Moderna) to BNT162b2 (Pfizer-BioNTech) for a COVID-19 breakthrough infection was 0.535, indicating a lower risk with Moderna compared to Pfizer-BioNTech [38]. Additionally, JNJ-78436735 showed an adverse event incidence two to eight times higher than that of the other vaccines in certain symptoms like pruritus, injection site erythema, and swelling [37]. Moreover, the incidence of hyperhidrosis after mRNA-1273 was more than three times higher than after other vaccines [38]. In terms of severe adverse events, the incidence per 100,000 people after vaccination with JNJ-78436735 was 2.18, which was higher compared to mRNA-1273 and BNT162b2 [38]. These findings suggest that while all vaccines have shown efficacy against COVID-19, there are variations in adverse event profiles between the JNJ-78436735 vaccine and the Moderna vaccine, with differences in both breakthrough infection risk and adverse event incidence.

When comparing the Pfizer-BioNTech and Novavax COVID-19 vaccines, several key differences emerge. Pfizer-BioNTech and Moderna vaccines use messenger RNA (mRNA) technology, instructing the body’s cells to make proteins that trigger an immune response against COVID-19. On the other hand, the Novavax vaccine is a protein-based vaccine that injects spike proteins directly into the body, leading to the production of virus-fighting antibodies and T cells. In terms of efficacy, both vaccines have shown high effectiveness. Studies suggest that the Novavax booster was about 55% effective at preventing COVID symptoms, like mRNA vaccines. Regarding side effects, the Novavax booster appears to have a lower risk of causing myocarditis or pericarditis compared to mRNA vaccines, although it is not without risk. Side effects such as muscle fatigue and nausea were reported to be fewer in the first 48 h after vaccination with the Novavax vaccine compared to mRNA vaccines. Overall, both Pfizer-BioNTech and Novavax vaccines offer strong protection against severe illness, hospitalization, and death from COVID-19. The choice between them may depend on individual preferences, availability, and specific health considerations.

The Oxford-AstraZeneca vaccine, also known as Vaxzevria, uses a chimpanzee adenovirus to deliver genetic material that teaches the body to produce antibodies against COVID-19. On the other hand, Moderna’s vaccine, known as Spikevax, utilizes mRNA technology to achieve the same goal of antibody production without using any virus particles. In terms of efficacy against severe COVID-19, both vaccines have shown strong performance, with Pfizer and Moderna being slightly more effective against mild illness compared to AstraZeneca and Johnson & Johnson’s vaccines. Booster doses of Vaxzevria have been found to enhance protection against the Omicron variant, like the increased immune response seen with Pfizer and Moderna boosters. Moreover, both vaccines have similar side effects, including injection-site pain and flu-like symptoms such as fever and fatigue. AstraZeneca’s Vaxzevria has been associated with a rare condition called thrombosis, leading to temporary pauses in its rollout in some regions due to concerns over blood clots. However, regulatory agencies like the European Medicines Agency (EMA) and the World Health Organization (WHO) have affirmed the safety of the AstraZeneca vaccine. While both vaccines are effective in preventing severe COVID-19, Moderna’s Spikevax has
shown slightly higher efficacy against mild illnesses compared to AstraZeneca’s Vaxzevria. Additionally, AstraZeneca’s vaccine has been linked to rare cases of thrombosis, although overall safety has been confirmed by regulatory bodies.

When comparing JNJ-78436735 (Johnson & Johnson’s vaccine) and NVX-CoV2373 (Novavax’s vaccine), several key differences emerge. Novavax’s NVX-CoV2373 vaccine has reported an efficacy of 89.7% against COVID-19 infection, with an 86.3% effectiveness against the B.1.1.7 variant and 96.4% effectiveness against non-B.1.1.7 variants [39]. On the other hand, the JNJ-78436735 vaccine has shown lower efficacy compared to mRNA vaccines but still meets certain standards [40]. NVX-CoV2373 contains the full-length spike protein of SARS-CoV-2 along with Novavax’s Matrix-M adjuvant. In contrast, JNJ-78436735 contains the S protein, stabilized by certain modifications [39]. Novavax’s vaccine can be stored in a refrigerator, making it easier to distribute, while JNJ-78436735 has specific storage requirements that may impact distribution logistics. Novavax’s NVX-CoV2373 is authorized for use in individuals aged 12 and older, offering a non-mRNA option in the U.S., whereas JNJ-78436735 is produced by Johnson & Johnson but is no longer available [39]. Novavax’s NVX-CoV2373 vaccine demonstrates high efficacy, ease of storage, and availability for a broader age group compared to Johnson & Johnson’s JNJ-78436735 vaccine, which has specific modifications in its composition and is no longer available for administration.

When comparing the Oxford-AstraZeneca and Novavax COVID-19 vaccines, several key differences also emerge. The AstraZeneca vaccine is a vector vaccine, while Novavax is a protein subunit vaccine. In terms of efficacy, Novavax’s vaccine has been shown to be more than 96% effective in preventing mild and severe illness from the original coronavirus strain and around 55% effective against the South African variant. On the other hand, AstraZeneca’s vaccine has demonstrated effectiveness against symptomatic SARS-CoV-2 infection after the first dose, with an efficacy of 84% and 61% after the second dose [41]. Additionally, Novavax’s vaccine can be stored in a refrigerator, making it easier to distribute, while AstraZeneca’s vaccine has faced concerns regarding its effectiveness against certain variants and potential side effects. Novavax’s protein subunit vaccine has shown high efficacy against the original strain but lower efficacy against certain variants, while AstraZeneca’s vector vaccine has demonstrated good effectiveness after two doses but raised some concerns regarding its overall efficacy and potential side effects.

The comparison between JNJ-78436735 (Johnson & Johnson) and Pfizer-BioNTech vaccines reveals some key differences in adverse events (AEs) and efficacy. According to the research, JNJ-78436735 showed an increased incidence of adverse events compared to Pfizer-BioNTech, particularly in symptoms like pruritus, injection site erythema, and injection site swelling. On the other hand, Pfizer-BioNTech had a higher incidence of hyperhidrosis compared to other vaccines. In terms of severe AEs, the incidence after vaccination with JNJ-78436735 was higher than after Pfizer-BioNTech, with 2.18 severe AEs per 100,000 people for JNJ-78436735 compared to 0.75 for Pfizer-BioNTech. Additionally, the study highlighted that younger age, female sex, and having had COVID-19 before vaccination were associated with greater odds of adverse effects [38].

When comparing the JNJ-78436735 and AZD1222 COVID-19 vaccines, several key differences and similarities also emerge from the provided sources. As for vaccine composition, JNJ-78436735 contains the S protein, which is stabilized by certain modifications [39]. AZD1222, on the other hand, is a viral vectored vaccine that includes information for the wild-type SARS-CoV-2 spike protein [42]. As for efficacy and safety, the efficacy of JNJ-78436735 was reported to be 70.4% without severe cases or hospitalizations after the second dose [42]. Preliminary data suggested that AZD1222 could reduce virus transmission and showed a good safety profile with mild adverse effects [42]. As for clinical trials, AZD1222 underwent Phase 3 trials with interim results known by December 2020, involving around 30,000 participants [39]. JNJ-78436735 was part of a Phase 1/2 trial where mild adverse reactions were reported by participants, with no serious adverse reactions documented [42]. As for administration, both vaccines require multiple doses for full vacci-
AZD1222 has a dosing schedule of two doses separated by about a month and given intramuscularly [39]. The study on adverse effects after COVID-19 vaccination reported that after two doses of BNT162b2 or mRNA-1273 or one dose of JNJ-78436735, 80.3% of participants reported adverse effects [43]. While both JNJ-78436735 and AZD1222 have shown efficacy and safety profiles in their respective trials, they differ in their composition and specific outcomes. JNJ-78436735 demonstrated effectiveness in preventing severe cases, while AZD1222 showed potential in reducing virus transmission and maintaining a good safety profile.

The comparison between the BNT162b2 (Pfizer/BioNTech) and mRNA-1273 (Moderna) COVID-19 vaccines reveals several key differences. Studies have shown that mRNA-1273 induces higher anti-spike and anti-RBD antibody levels compared to BNT162b2, with a greater proportion of individuals reaching convalescent serum levels for these antibodies [44]. Additionally, mRNA-1273 has demonstrated higher effectiveness against Delta-related hospitalization and fatality compared to BNT162b2, with both vaccines showing robust effectiveness against severe disease caused by the Delta variant [45]. However, the BNT162b2 vaccine has been associated with a higher risk of documented SARS-CoV-2 infection, symptomatic COVID-19, hospitalization, ICU admission, and death compared to mRNA-1273 over a 24-week period [46]. While both vaccines are highly effective against COVID-19, mRNA-1273 appears to elicit stronger antibody responses and demonstrate higher effectiveness against severe outcomes related to the Delta variant when compared to BNT162b2.

In addition, the three-dimensional (3D) structure of epitopes is essential for functional analysis and structure-based vaccine design [47]. However, the bottleneck of experimental methods such as X-ray crystal diffraction is that a series of analysis processes, including protein preparation, require a lot of time and effort. AlphaFold2 and RoseTTAFold, which utilize AI (artificial intelligence), have been successively reported as protein structure prediction technologies that solve this problem [48]. Both are methods that use large-scale protein structure information from the Protein Data Bank as training data to predict protein folding structures only from amino acid sequence information [49]. By using complementary methods with experimental methods, it is expected that protein structure analysis and its application areas will further develop (Figure 1). In this review, our purpose is to summarize the structure-based vaccine design and mechanisms of SARS-CoV-2 infection.
Figure 1. Vaccine development and 3D structure analysis of the protein of the virus. Conventional (X-ray crystal diffraction and Molecular Dynamics Stimulation) and newest (Rose TTAFold and AlphaFold2) analyses.

2. Vaccination with Nanoparticles (LNP) and Extracellular Vesicles as New DDS

In addition, vaccination with the mRNA-lipid nanoparticle (LNP) vaccine against COVID-19 is underway around the world [50–52] (Figures 2 and 3). This drug requires frozen storage during transportation and at inoculation facilities, which poses a challenge to rapid vaccination dissemination [53,54]. As a structural feature, the mRNA is encapsulated with water in vesicles consisting of cationic lipids and a cholesterol membrane inside the LNP [50]. Therefore, the key to stabilization is how to suppress mRNA hydrolysis in this aqueous environment. Generally, since mRNA is most stable in a weakly basic environment (pH from 7 to 8), it is important to adjust the pH inside the LNP [55–58]. Furthermore, lyophilization of the formulation is a more effective method, and it is necessary to ensure that the efficacy is not diminished after resuspension [59,60].

Meanwhile, modified extracellular vesicles (EVs) loaded with nanobodies against the spike protein of SARS-CoV-2 and interferon-beta, a cytokine with antiviral effects, have been reported [61–64]. EVs are biological substances with a heterogenous lipid bilayer structure that are secreted from almost all living cells and broadly classified into three types: exosomes, microvesicles, and apoptotic bodies, depending on the intracellular production mechanism [65–67]. EVs contain substances such as nucleic acids, proteins, and lipids derived from the producing cells and function as an intercellular transport carrier for these substances. Therefore, EVs can be considered endogenous delivery carriers and a safe and efficient drug delivery system [67,68]. Therefore, there are high expectations for the development of drug delivery systems (DDSs) using EVs, including small EVs. However, in order to achieve this, there are issues that need to be resolved, such as issues related to EV production such as selection of EV-producing cells, development and optimization of a method for recovering EVs released into the culture supernatant of production cells from the culture medium and how to preserve it, establishing methods for loading drugs into EVs, and understanding and controlling the internal dynamics of EVs [69–72].
**Figure 2.** mRNA-lipid nanoparticle (LNP) vaccine.

**Figure 3.** The molecular interaction between the vaccine-induced immune response and the SARS-CoV-2 virus, including how T cells, B cells, and antibodies interact with the virus’s spike protein.

EVs play a significant role in SARS-CoV-2 infection by facilitating the spread of the virus and influencing the immune response. EVs released from virus-infected cells contain viral components and receptors that make recipient cells susceptible to infection. These EVs can transfer viral components like CD9 and ACE2, aiding in virus docking and entry.
into cells. Studies have shown that EVs can carry many virus particles, contributing to the transmission of SARS-CoV-2 [73–75]. Furthermore, EVs released from SARS-CoV-2-infected cells are involved in reprogramming the proteome and transcriptome of host cells, promoting the viral life cycle, and sustaining infection. The virus utilizes cellular metabolic pathways for entry, replication, and egress, leading to altered serum profiles and abnormal glucose homeostasis. In severe cases, immune-pathological factors can result in multi-organ damage due to the reprogramming of host systems by the virus [76]. Understanding the role of EVs in SARS-CoV-2 infection is crucial for developing therapeutic strategies. EV-based approaches such as inhibiting EV biogenesis, utilizing EV therapy, developing EV-based drug delivery systems, and creating EV-based vaccines are being explored as potential treatments for COVID-19 [73].

Additionally, during viral infection, large amounts of EVs are secreted from infected cells, which function as a communication network between cells and coordinate the response to infection. Furthermore, individuals vaccinated with mRNA vaccines acquire circulating EVs containing the SARS-CoV-2 spike glycoprotein by day 14 post-vaccination [76,77]. Additionally, antibodies to the spike glycoprotein are absent from the circulation 14 days after the first vaccination [78,79]. However, after the second vaccination, the number of spike glycoprotein-containing EVs in the blood increased by up to 12 times [80,81]. Also, antibodies first appeared on day 14. EVs display spike glycoproteins on their surface, which promotes antibody production [82–84]. Furthermore, when mice were exposed to EVs from vaccines, they produced antibodies against the spike glycoprotein [85,86]. Interestingly, after peak expression, the number of spike glycoprotein-containing EVs in the circulation decreases over time with decreasing antibody levels against the spike glycoprotein [64,83]. EVs exist as part of the mRNA decay machinery under stress conditions, in close association with stress granules and P bodies [87]. Under the condition of vaccine-mRNA-guided translation, which can be termed “over-reliance on cap-dependent translation”, there is an apparent resistance to the promotion and assembly of large decapping complexes and thus to physiological mRNA degradation processes [88]. This means that the fate of specific synthetic mRNAs, which was determined by a common cellular strategy for mRNA turnover involving the messenger ribonuclein proteins (mRNP), has been omitted [89]. Furthermore, under conditions where the synthetic mRNA for the SARS-CoV-2 vaccine is overly dependent on cap-dependent translation, many natural mRNAs that retain significant internal ribosome entry sites (IRESs) and specific methylation (m6A) in their structure favor cap-independent translation, which is strongly associated with disruption of mRNA quality control mechanisms [90]. Thus, significant deadenylated mRNA products derived from mRNA metabolism or decay are directly linked to cargo within EVs [91].

Due to the high demand for gene expression, high concentrations of certain miRNAs are included in EVs via P-bodies. Additionally, under conditions where SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) molecular vaccination produces overwhelming SARS-CoV-2 spike glycoprotein, a significant proportion of the overproduced intracellular spike glycoprotein is transported via EVs [3,92–94]. Spike, the gene product of SARS-CoV-2, can modify host EV cargo, which can be transported to distant, uninfected tissues and organs, and initiate devastating immune cascades within the central nervous system. By exposing human HEK293T cells to the SARS-CoV-2 spike gene plasmid, the synthesis of spike glycoprotein is induced within the cell, which releases large amounts of EVs containing the spike glycoprotein along with specific miRNAs [89,95]. Furthermore, long-distance cell-to-cell communication mediated by EVs may be a mechanism by which neurological symptoms manifest in severe cases of COVID-19 [96,97]. Through inhibition of ubiquitin-specific peptidase 33 (USP33) and IRF9, two key proteins that control this pathway, two miRNAs, miR-148a and miR-590, specifically inhibit type I interferon signaling [98]. In microglia exposed to EVs extracted from HEK293 cultures, cellular expression of USP33 (ubiquitin-specific peptidase 33) is reduced by approximately 50% and that of IRF9 (interferon regulatory factor 9) is reduced by approximately 60% via miR-148a
and miR-590, respectively [98,99]. USP33 removes ubiquitin from IRF9, protecting it from degradation [98]. Therefore, the two miRNAs jointly interfere with IRF9, blocking the receptor response to type I interferon [99,100]. Also, EVs mediate neutralizing antibody-resistant transmission of SARS-CoV-2 by encapsulating large amounts of live virus particles within SARA-CoV-2-induced EVs and can reinfect naïve cells independent of the receptors and cofactors [82,86]. Therefore, it will be important to understand the infection mechanisms of SARA-CoV-2 via receptor-dependent and receptor-independent uptake into cells and to model the infection processes based on 3D structural features at interaction sites.

3. Spike Proteins and the Receptors of SARS-CoV-2

A computer simulation of one of the spike proteins of SARS-CoV-2 shows that sugar chains are tightly wrapped around the spike protein that protrudes from the surface of the virus [101,102]. They coat the outer protein with sugar chains, fooling our immune system’s surveillance eyes like a wolf in sheep’s clothing. Based on the structural and genetic data of SARS-CoV-2, the sugar coat was visualized using a supercomputer to render each atom of the sugar chain. There are bare loops, not covered by sugar chains, that protrude from the top of the protein. This is the receptor binding domain (RBD), one of three sections that the virus’s spike protein uses to bind to receptors on human cells. In the simulation, when the RBD is lifted and placed above a cloud of sugar chains, the two-sugar chain quickly approaches and fixes the RBD in place. Furthermore, when these sugar chains are mutated in a computer model, the RBD collapses [102–104]. Mutating these two sugar chains reduces the ability of the spike protein to bind to human cell receptors [105,106]. Cutting out these two sugar chains may weaken the infectivity of the virus, but there is no way to do so yet [107–109]. By understanding the infection process in this way, it could help improve treatments and vaccines and understand why new variants are more transmissible [110]. Researchers have uncovered SARS-CoV-2’s ability to bind to human cells with remarkable strength, as well as key adaptive abilities that help it hide inside cells [103,111,112]. As new virus particles exit the cell, they undergo certain important processing steps, preparing them to infect more human cells. These are just some of the tools that have given this virus its ability to spread rapidly and claim so many lives. That is why it is so hard to get this virus under control. When this virus enters the cell, the endoplasmic reticulum inside the cell changes its shape into a foam-like double-membrane vesicle, which serves as a hiding place for more TNA viruses to replicate and translate [113]. The newly created molecules are assembled into a complete virus particle, which is then released outside the cell via the Golgi complex, or lysosomes.

The outside of the SARS-COV-2 virus particle is a lipid membrane-encased envelope, and 24 to 40 spike proteins are randomly arranged on its surface [114–117]. While the membrane fusion proteins that protrude from the envelope of viruses such as influenza are relatively rigid, the spike protein of SARS-CoV-2 is extremely flexible and has three “joints” [103,118]. With this structure, the spike protein can flap, wobble, and rotate, facilitating probing cell surfaces and binding multiple spike proteins to a single human cell. Although there is no similar experimental data on other coronaviruses, we consider this characteristic to be shared because the spike protein sequence is highly evolutionarily conserved [119–121]. The RBD of the SARS-CoV-2 spike protein binds to the human angiotensin-converting enzyme 2 (ACE2) receptor, which is found on the surface of most cells in the throat and lungs. SARS-CoV, the virus that causes SARS, also binds to the ACE2 receptor, but the binding strength of SARS-CoV-2 is two to four times stronger [122]. This is because multiple changes occur in the RBD, stabilizing the virus binding hotspot [123–125]. Variants of SARS-CoV-2 that are of particular concern often have mutations in the S1 subunit of the spike protein [19,126,127]. The S1 subunit has an RBD and is responsible for binding to the ACE2 receptor [128–131]. For example, in the alpha strain, the spike protein sequence has changed in 10 places, making it easier for the RBD to stay in the “up-type” configuration, which makes it easier for the virus to enter cells by increasing the ability of RBD to bind to ACE2 and escape from immune system surveillance [132–134].
EVs play a crucial role in transporting the spike glycoprotein of SARS-CoV-2, impacting antibody responses and potentially patient outcomes during COVID-19 [83,135]. These EVs can carry the S protein, acting as decoy targets for neutralizing antibodies and reducing their effectiveness in blocking viral entry [83]. Understanding the transportation of spike glycoprotein via EVs is vital as it sheds light on the complex mechanisms of SARS-CoV-2 infection, particularly how EVs can modulate immune responses and promote viral infections [73,126]. Additionally, research has shown that EVs containing the S protein can be used as a tool to mimic SARS-CoV-2 interactions with host cells and potentially inhibit viral infection [136,137]. The incorporation of viral proteins into EVs has been explored for therapeutic purposes, highlighting the potential of EV-based formulations in clinical trials for treating viral infections [135,137]. Studying the role of EVs in viral infections like COVID-19 provides valuable insights into disease pathogenesis and potential therapeutic strategies [138].

4. Fusion of the Viral Membrane with the Host Cell Membrane

When the viral spike protein binds to ACE2, other proteins on the surface of the host cell begin a process that fuses the viral membrane with the host cell membrane [118,139,140]. SARS-CoV uses one of two types of host proteases to enter cells [103,112,141]. One is an enzyme called transmembrane protease, serine 2 (TMPRSS2), and the other is an enzyme called cathepsin L [142–144]. Although TMPRSS2 allows for faster entry, SARS-CoV often uses cathepsin L to enter cells via endosomes. However, virus particles that enter cells through this route are captured by antiviral proteins [145,146]. SARS-CoV-2 differs from SARS-CoV in that it efficiently utilizes TMPRSS2, which is abundant on the outside of respiratory cells [147,148]. TMPRSS2 first cleaves a specific site on the S2 subunit of the spike protein [149,150]. This exposes the hydrophobic amino acid sequence of the spike protein [151]. These amino acids quickly penetrate the nearest host cell membrane [139,152]. Next, the long spike protein folds, causing the virus and cell membrane to fuse together, like closing a zipper [118,136,153]. The virus fuses with the cell membrane and releases its genome directly into the cell. SARS-CoV-2 enters cells in a spring-locked manner, allowing it to infect faster than SARS-CoV and avoid being taken up by endosomes [118,154]. The anti-malarial drug chloroquine looked promising as a COVID-19 treatment in early laboratory studies but failed in clinical trials [155]. The reason is that SARS-CoV-2 uses TMPRSS2 to quickly invade cells [112,118,148]. The cells used in early research only had access through endosomes using cathepsin L [142]. SARS-CoV-2 does not use endosomes when it infects the human respiratory tract and multiplies, so chloroquine, which destroys endosomes, had no effect. The finding also shows promise for protease inhibitors as therapeutic agents that prevent viruses from using proteases such as TMPRSS2 and cathepsin L to enter host cells. Camostat mesylate, one of the TMPRSS2 inhibitors, can block the virus from entering lung cells [156]. However, early clinical trials failed to improve patients’ outcomes.

5. RNA Genome and Translation in SARS-CoV-2

When SARS-CoV-2 releases its RNA genome into a cell, ribosomes in the cytoplasm translate two sections of the viral RNA into a long sequence of amino acids. This long amino acid is cleaved into 16 different proteins, which contain many proteins involved in RNA synthesis, so more viral RNA is made [157]. These RNAs encode a total of 26 known viral proteins, including the spike protein, other structural proteins, and other accessory proteins needed to make new virus particles [158–160]. The virus thus begins making copies of its own mRNA, but it requires the host’s cellular machinery to translate the mRNA and make viral proteins [161]. Coronaviruses hijack the host’s cellular machinery in a variety of ways [162,163]. There are three mechanisms by which SARS-CoV-2 suppresses host mRNA translation to prioritize translation of its own mRNA. None of these mechanisms are unique to this virus, but their combination, speed, and magnitude of effects are unparalleled. In the first mechanism, the virus eliminates competitors. Nsp1 (nonstructural protein 1), one of the first viral proteins translated in the cell, recruits host proteins to systematically
chop up all host cell mRNA that is not virus-tagged [164]. In the second mechanism, the virus reduces protein translation in infected cells by an overall 70%. Again, Nsp1 plays a key role, physically blocking the ribosome’s mRNA channel and preventing mRNA from entering [165]. The little remaining translation capacity is used to translate viral RNA. In a third mechanism, the virus shuts down the cell’s alarm system. This prevents host cell mRNA from exiting the nucleus. This prevents host cell mRNA from exiting the nucleus. Nsp1 is responsible for preventing this, and Nsp1 blocks the nuclear membrane pores so that nothing can escape from the nucleus [166]. Virus-infected cells release small amounts of interferon because gene transcripts cannot leave the nucleus. SARS-CoV-2 is particularly capable of shutting down alarm systems, and infection with this virus releases significantly less interferon than other respiratory viruses, such as SARS-CoV and respiratory syncytial virus [167–170]. SARS-CoV-2 is a very fast-transmitting virus that has a unique ability to prevent the immune system from recognizing and dealing with it during the early stages of infection [171,172]. By the time the immune system realizes the virus is present, the amount of virus can be so large that more immune-reactive proteins than normal are released into the bloodstream all at once, causing more harm than good [173,174]. Some severely-ill COVID-19 patients are not only damaged by the virus but also by an exaggerated immune response to the virus. Some proven treatments suppress this immune response.

After hijacking the host cell’s translation machinery, the virus begins to remodel the cell [175]. They significantly alter the inside and outside of cells to suit the needs of the virus. First, some of the newly created viral spike proteins move to the cell surface and protrude out of the host’s cell membrane [176]. Therefore, it activates calcium ion channels in the host cell, causing a lipid coating to be expelled from the outside of the cell. This capsule is the same one found in types of cells that naturally fuse, such as muscle cells. Eventually, infected cells fuse with neighboring cells that express ACE2 and grow into one giant respiratory cell with up to 20 nuclei, which is termed syncytia [177,178] (Figure 4). Syncytia is formed by infection with SARS-CoV-2, human immunodeficiency virus (HIV), herpes simplex virus, etc., but not with SARS-CoV [177,179]. The formation of syncytia also allows infected cells to survive longer and produce more virus particles [179,180]. Additionally, some cells infected with SARS-CoV-2 can even form syncytia with lymphocytes, one of the body’s immune cells [178,181]. This has long been known as a tumor immune escape mechanism. The findings suggest that cells infected with SARS-CoV-2 evade immune system surveillance by clinging to and merging with nearly immune system scouts [182–184]. Thus, the SARS-CoV-2 attack is not temporary but persistent.
6. Importance of Structural Biology in SARS-CoV-2

Structural biology plays a crucial role in understanding SARS-CoV-2 and developing potential therapies for COVID-19. The virus encodes 29 proteins involved in various stages of its life cycle, such as viral entry, replication, transcription, assembly, and release. These proteins can act individually or in complexes with host cellular factors. Studies on the structures of these proteins provide insights into the mechanisms underlying viral infection and potential therapeutic targets. Notably, the S protein, papain-like protease (PLpro), main protease (Mpro), and viral RNA-dependent RNA polymerase (RdRP) are highlighted as important targets for therapeutic intervention [185, 186]. Structural biology has paved the way for the development of antiviral drugs and vaccines targeting specific viral proteins, offering hope for effective treatments against COVID-19 [187, 188].

7. Intracellular Changes with Virus Infections

Many more changes are occurring inside the cell. Inside cells, there is a network structure called the endoplasmic reticulum (ER), which consists of flat membranes and is involved in protein synthesis and transport [189]. Like other coronaviruses, SARS-CoV-2 inflates its long, thin endoplasmic reticulum like a soap bubble into a spherical double-membrane vesicle (DMV), which acts as a “hidden” place where viral RNA is replicated and translated without being detected by the innate immune system within the cell [190, 191]. The host protein TMEM41B is needed to mobilize cholesterol and other lipids to expand the endoplasmic reticulum membrane so that all the parts of the virus can fit inside the membrane [192, 193]. Nsp3 (nonstructural protein 3) creates a crown-shaped pore in the DMV wall and exports newly produced viral RNA [194, 195]. Most viruses that have envelopes use part of the cell membrane near the edge of the cell to assemble the envelope and exit. However, in the case of coronaviruses, the newly created proteins take a different route. Coronavirus is transported out of cells via the Golgi complex [196–198]. The Golgi complex is a cellular organelle that acts like a post office, wrapping molecules in a membrane and shipping them to other locations within the cell. Coronaviruses form a lipid envelope from the membranes of the Golgi complex, and the newly formed virus particles enter Golgi vesicles, are transported to the cell surface, and are expelled from the cell [198–201]. Coronaviruses can also exit cells through lysosomes. Therefore, blocking the
secretory pathway that utilizes the Golgi complex does not affect the amount of infectious virus released [202]. Viral proteins bud into the endoplasmic reticulum, form an envelope, and then hijack lysosomes to exit the cell [203–205]. Exiting the cell via the Golgi complex or lysosome takes longer and is less efficient than budding from the cell membrane. One event that occurs when a virus leaves a cell makes a new virus a “contagious monster”. A five-amino acid sequence in the virus is cleaved so that when it leaves, it is ready to invade its next target [149,206]. In other coronaviruses, there is only one amino acid, arginine, at the junction of the S1 and S2 subunits of the spike protein, whereas SARS-CoV-2 has five amino acids arranged in the following order: proline (P), arginine (R), arginine (R), alanine (A), and arginine (R), which is essential for entry into lung cells [207–210]. A host cell protease called furin recognizes and cleaves this PRRAR sequence, and this cleavage is essential for the virus to efficiently invade human lung cells. Also, ferrets infected with a cultured strain of SARS-CoV-2 that has naturally lost the furin cleavage site shed fewer virus particles than ferrets infected with a pandemic strain and do not spread infection to nearby animals [211]. Also, coronaviruses that do not have a modified furin cleavage site invade human airway cells faster than those that have a modified furin cleavage site. Furin cuts the cleavage site when the virus particle is assembled or just before it is released [148,212]. Viruses exit the cell via the Golgi complex or lysosomes because the assembled virus moves into organelles that are rich in furin [213,214]. Furin loosens the spike protein by breaking the bond between its S1 and S2 subunits [103,215]. When the virus in this state invades the next cell, it undergoes a second cleavage by TMPRSS2, exposing the hydrophobic region, which is quickly buried in the host cell membrane.

If the spike protein has not been previously cleaved by furin, it will not be cleaved by TMPRSS2 either, or it will enter cells slowly through the endosomal route [148,216]. The more basic the amino acid sequence at the furin cleavage site, the more efficiently furin can recognize and cleave it. The furin cleavage site in the mutant strain reduces the acidity of the amino acid sequence [148,217,218]. Thus, this further increased the infectivity of the mutant strain. Being more susceptible to furin cleavage means more spike proteins are ready to enter human cells [148,216,219]. In SARS-CoV, less than 10% of the spike protein is ready to enter human cells, but in SARS-CoV-2, this increases to 50%, and more than 75% of the spike protein is ready to enter humans in highly infectious variants [129,133,220]. Important factors include the number of ACE2 receptors required for one spike protein to bind to a host cell, the cleavage site of the S2 subunit, the exact timing at which the cleavage site of the S2 subunit is cleaved by TMPRSS2, and the number of spike proteins required for virus and cell membrane fusion [118]. Most of the mutations in the data have been related to how efficiently the virus spreads rather than how much damage it does to its host [76,220,221].

As for the role of EVs in COVID-19 vaccination and immune response, mRNA-based vaccines targeting the SARS-CoV-3 s protein have been authorized for COVID-19 vaccination, showcasing the significance of EVs in vaccine delivery and immune system activation [222,223]. Additionally, research has explored the potential of engineered EVs with pathogen proteins as promising alternatives to lipid nanoparticle (LNP)-mRNA vaccines, indicating a growing interest in utilizing EVs for vaccine development [224]. The increase in the number of spike glycoprotein-containing EVs in the blood in S protein presence post-vaccination has been observed in studies related to both natural infection and vaccination against COVID-19 [79,81,225]. The S protein of SARS-CoV-2 has the ability to alter the cargo of host EVs, which can then travel to distant, uninfected tissues and organs. The process can trigger harmful immune responses within the central nervous system. The S protein’s interaction with host cells plays a crucial role in the immune evasion mechanisms employed by the virus, contributing to its ability to evade the host immune system [226,227]. EVs play a crucial role in mediating the transmission of SARS-CoV-2 by encapsulating live virus particles, enabling them to evade neutralizing antibodies and infect cells. These vesicles aid in the escape of viruses from antibody neutralization,
facilitating productive infections [74]. Specifically, neutralizing antibodies targeting the S protein of SARS-CoV-2 can prevent the virus from binding to ACE2 receptors, thereby blocking viral fusion and infection [82]. Understanding the structural aspects of SARS-CoV-2, particularly the spike glycoprotein and receptor-binding domain, is essential for developing effective strategies against COVID-19 [228]. Variants of SARS-CoV-2, such as those carrying mutations like L452R in the S protein, impact transmission, infectivity, and antibody neutralization [229]. Some variants, like the B.1.1.529 VOC, exhibit resistance to many monoclonal antibodies, highlighting the challenges posed by viral evolution in evading immune responses [230]. SARS-CoV-2, the virus responsible for COVID-19, has developed various strategies to evade the immune system and facilitate infection. These evasion mechanisms include inhibiting the communication of interferons (IFNs), antagonizing IFNs, and interfering with multiple antiviral surveillance systems. Thus, the virus can escape immune surveillance and enhance its ability to infect cells. The S protein of SARS-CoV-2 is a key target for our immune system, and the virus has evolved ways to manipulate this interaction to its advantage [182,231,232].

Future extensions and applications of novel therapeutic strategies for COVID-19 vaccines are crucial in the ongoing fight against the pandemic. Recent developments include the FDA extending the expiration date of certain lots of the Moderna COVID-19 vaccine from 9 to 12 months when stored properly. Additionally, Pfizer and BioNTech received U.S. FDA approval for their COVID-19 vaccine, COMIRNATY®, for active immunization against COVID-19. The FDA also updated the emergency use authorization for the Pfizer-BioNTech COVID-19 vaccine to support an extension of its shelf life.

The development of vaccines targeting the spike glycoprotein of SARS-CoV-2 has been a crucial focus in the fight against COVID-19. However, there are several limitations and challenges associated with these vaccines, as follows:

1. As for the duration of protection, the longevity of protection provided by current vaccines against SARS-CoV-2 is still uncertain. Antibody levels can decrease rapidly after infection, raising concerns about the long-term effectiveness of vaccines [2,233].

2. As for variants and mutations, the emergence of highly transmissible variants poses a significant challenge to vaccine efficacy. Mutations in the virus, especially in the S protein, can impact the effectiveness of existing vaccines against new variants [81].

3. As for glycosylation patterns, variations in the glycosylation patterns of the S protein can affect vaccine efficacy. Different degrees of glycosylation can influence binding reactivity to antibodies and the induction of immune responses, potentially impacting vaccine effectiveness [9].

4. As for cellular immune response, while neutralizing antibodies are a primary target for vaccines, the importance of cellular immune responses, particularly T-cell immunity, in controlling SARS-CoV-2 infection is significant. Understanding and harnessing these cellular responses are crucial for comprehensive vaccine development [81].

While vaccines targeting the spike glycoprotein have shown remarkable progress in combating COVID-19, ongoing research is essential to address these limitations and enhance the effectiveness and durability of vaccination strategies against SARS-CoV-2 [2,41,81,233].

8. Conclusions

Individuals vaccinated with mRNA vaccines acquire circulating EVs containing the SARS-CoV-2 spike glycoprotein by day 14 post-vaccination. After the second vaccination, the number of spike glycoprotein-containing EVs in the blood increased by up to 12 times. Spike, the gene product of SARS-CoV-2, can modify host EV cargo, which can be transported to distant, uninfected tissues and organs, and initiate devastating immune cascades within the central nervous system. EVs mediate neutralizing antibody-resistant transmission of SARS-CoV-2 by encapsulating large amounts of live virus particles within SARS-CoV-2-induced EVs and can reinfect naïve cells independent of the receptors and
cofactors. Also, cells infected with SARS-CoV-2 evade immune system surveillance by clinging to and merging with nearly immune system scouts.

The policy implications of novel therapeutic strategies for COVID-19 vaccines involve the urgent need to enhance existing prevention and control measures by focusing on the development of innovative vaccines and drugs. Researchers are actively exploring new therapeutic approaches to combat COVID-19, aiming to improve treatment outcomes and reduce the impact of the virus. Efforts are underway globally to develop safe and effective vaccines and therapeutics, highlighting the importance of equitable distribution to ensure widespread access to these critical interventions.

There is a call for next-generation COVID-19 vaccines to address evolving challenges. Various approaches to future vaccine development are being explored, offering hope for more effective and adaptable solutions to combat the virus. The U.S. COVID-19 vaccination program has transitioned towards a more commercialized market, indicating a shift in strategies and opportunities for innovation in vaccine distribution and development. These advancements underscore the importance of continuous research and adaptation to enhance vaccine efficacy and accessibility in the global fight against COVID-19.

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