Review

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Review

Causes and Consequences of Coronavirus Spike Protein Variability

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Abstract: Coronaviruses are a large family of enveloped RNA viruses found in numerous animal species. They are well-known for their ability to cross species barriers and have been transmitted from bats or intermediate hosts to humans on several occasions. Four of the seven human coronaviruses (hCoVs) are responsible for approximately 20% of common colds (hCoV-229E, -NL63, -OC43, -HKU1). Two others (SARS-CoV-1 and MERS-CoV) cause severe and frequently lethal respiratory syndromes but have only spread to very limited extents in the human population. In contrast the most recent zoonotic hCoV, SARS-CoV-2, while exhibiting intermediate pathogenicity, has a profound impact on public health due to its enormous spread. In this review, we discuss which initial features of the SARS-CoV-2 Spike protein and subsequent adaptations to the new human host may have helped this pathogen to cause the COVID-19 pandemic. Our focus is on host forces driving changes in the Spike protein and their consequences for virus infectivity, pathogenicity, immune evasion and resistance to preventive or therapeutic agents. In addition, we briefly address the significance and perspectives of broad-spectrum therapeutics and vaccines.

Keywords: SARS-CoV-2; sarbecoviruses; spike; mutation; manifestation; immune evasion; zoonoses

Introduction

Coronaviridae represent a large family of diverse enveloped single-strand RNA viruses that received their name from the crown-like appearance of their Spike surface glycoproteins. Bats and rodents are considered the reservoir species of most coronaviruses[1]. However, coronaviruses are notorious for their ability to cross species barriers[2,3]. Consequently, they have been detected in many animal species and were successfully transmitted to humans at least seven times[4]. Four of the seven human coronaviruses (229E, NL63, OC43 and HKU1) cause mild respiratory infections and are responsible for about 20% to 30% of common colds[5,6]. The remaining three cause severe respiratory diseases, with lethality rates ranging from approximately 1% (SARS-CoV-2) to around 10% (SARS-CoV-1) and 40% (MERS-CoV)[7,8]. SARS-CoV-2 was most likely transmitted to humans from bats or an intermediate host at the end of 2019 and caused the COVID-19 pandemic[6,9–12]. To date, SARS-CoV-2 has infected more than 800 million people. Reliable and affordable tests, as well as effective vaccines and therapeutics, have been developed in an amazingly short time. However, emerging new SARS-CoV-2 variants that at least partially escape immunity generated by previous infection or vaccination and/or show increased replication and transmissibility continue to circulate in the human population.

The overall high diversity of different members of the coronavirus family and the ongoing emergence of new SARS-CoV-2 variants through mutation and recombination give the impression that coronaviruses mutate and diversify very rapidly. However, mutation and diversification rates of coronaviruses are actually much lower compared to other RNA viruses, such as human immunodeficiency virus (HIV) or hepatitis C virus (HCV)[13,14]. The reason for this is that the coronavirus polymerase has a proofreading activity, which rectifies errors during replication—a trait rather rare in the realm of RNA viruses[15,16]. For SARS-CoV-2 mutation rates of 1.3×10^{-6} per base and

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infection cycle have been reported[14]. Thus, coronaviruses are at the low end of the spectrum of 10^{-4} to 10^{-6} nucleotide substitutions per replication cycle reported for RNA viruses and approximating those of DNA viruses, which range from 10^{-6} to 10^{-8} [13]. Proof-reading activity also allows coronaviruses to possess one the largest genomes among RNA viruses, approximately 30 kilobases in length and encoding for about 30 proteins[17,18].

Mutations, recombination and ongoing replication are prerequisites for virus diversification. Usually, however, only changes providing a selection advantage for viral spread will be enriched and manifested in virus populations[19,20]. The forces driving fitness advantages vary and change upon zoonotic transmission. For example, it has been reported that SARS-CoV-2 initially evolved alterations increasing its replication and transmission fitness in the new human host[19,21]. After infection and/or vaccination of large parts of the human population, however, selection pressure to evade humoral immune responses increased and drove the emergence of new SARS-CoV-2 variants[19,21,22]. In some cases, initial changes allowing humoral immune evasion came at the cost of decreased infectivity but subsequent changes restored viral replication fitness[21,23]. The ongoing arms race between immune control and viral evasion drives the constant evolution of new SARS-CoV-2 variants.

As of December 2023, more than 13 billion COVID-19 vaccine doses have been administered (WHO COVID-19 dashboard) and most people have some immunity against SARS-CoV-2 through vaccination and/or previous infection. Thus, SARS-CoV-2 lost most of its fright, although it continuous to circulate and evolve within the human population. Thus, new questions arise, such as whether vaccines and therapeutics that are currently available or under development can protect us against newly emerging SARS-CoV-2 variants and future zoonoses of animal coronaviruses. Here, we address some of the factors driving the evolution of SARS-CoV-2 Spike proteins and their consequences for viral fitness and sensitivity to vaccines, neutralizing antibodies (nAbs), and Spike-targeting therapeutics. In addition, we discuss whether broad or even general protection against new SARS-CoV-2 variants and future zoonoses of animal coronaviruses may be feasible.

Early features of the SARS-CoV-2 Spike protein

The Spike proteins of different members of the coronavirus family show high sequence divergence[24]. SARS-CoV-2 is phylogenetically closely related to some bat CoVs, such as BANAL-20-52 and RaTG13 (Figure 1)[9,25,26]. The Spike protein of SARS-CoV-2 shows ~96% amino acid sequence homology to its closest bat relatives with most variations being located in the receptor binding domain (RBD) and the N-terminal domain (NTD) (Figure 2). Just like the Spike proteins of SARS-CoV-1 and hCoV-NL63, SARS-CoV-2 utilizes the angiotensin-converting enzyme 2 (ACE2) receptor for infection of human target cells[27–29]. Thus, ACE2 usage seems helpful but not essential for zoonotic transmission of coronaviruses. The Spike proteins of several coronaviruses detected in bats or intermediate host efficiently use human ACE2 for infection[30,31]. In comparison, the original RaTG13 Spike is poorly infectious in human cells but a single T403R change allows efficient usage of ACE2 receptors from various species including humans[31,32]. Most Spike proteins of related bat coronaviruses contain an R or K residue at position 403 of the viral Spike protein and are capable of infecting human cells. Thus, SARS-CoV-2 was most likely able to efficiently use human ACE2 immediately after cross-species transmission. A variety of alternative receptors have been reported to promote ACE2-independent entry into human cells but their relevance for SARS-CoV-2 replication and transmission in vivo is poorly understood[33].

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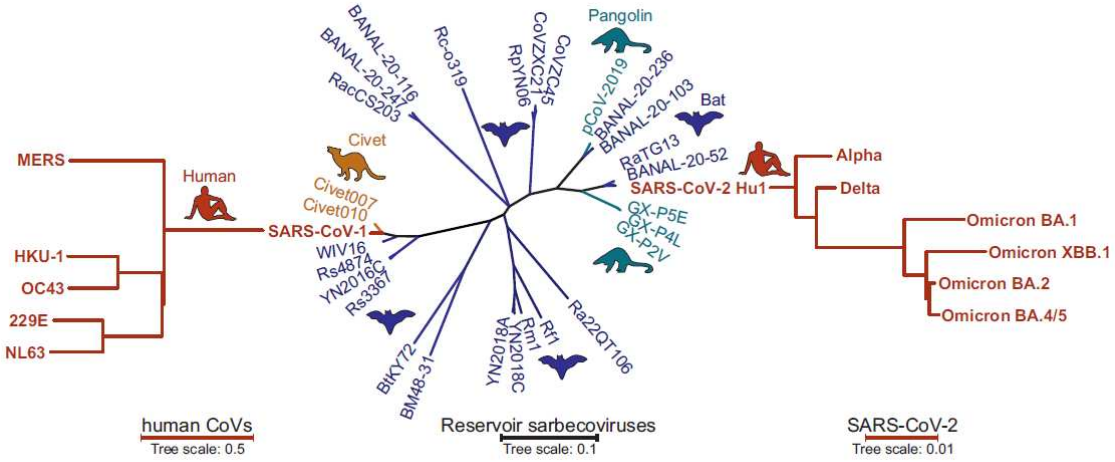

Figure 1. Phylogenetic relationship between human coronavirus (left), animal Sarbecovirus (middle) and human SARS-CoV-2 VOC Spike (right) amino acid sequences from the indicated viral strains or species. Host species are indicated by silhouettes: bat (blue), pangolin (turquoise), civet (orange) and human (red). Sequence identifiers are provided in supplementary Table S1.

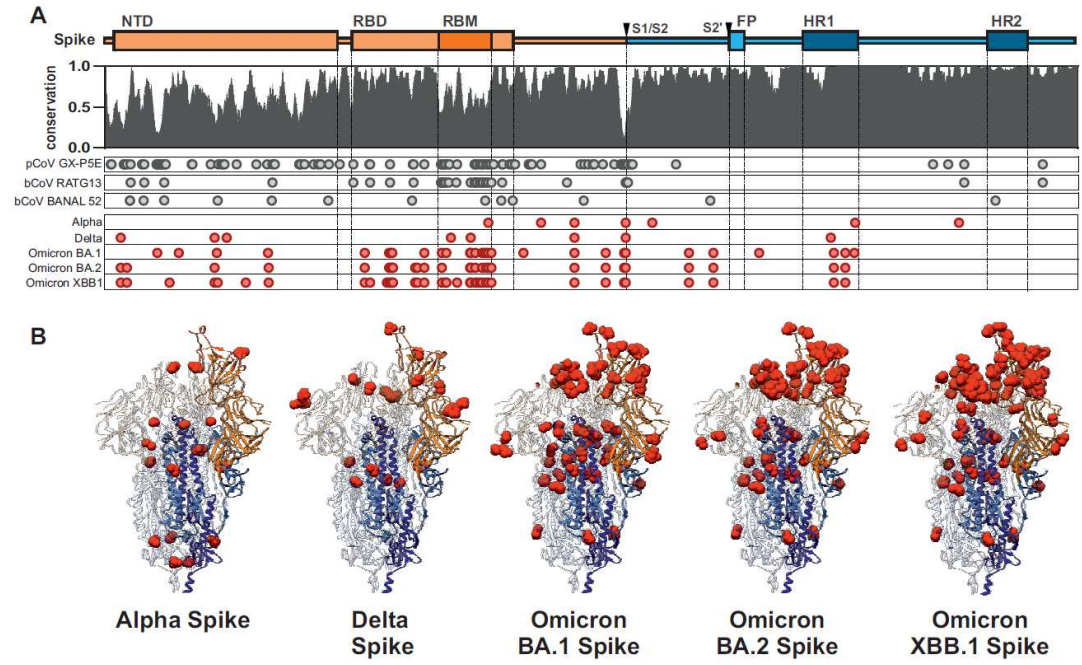

Figure 2. Conservation and mutation mapping of SARS-CoV-2 VOCs and Sarbecovirus Spike proteins. (A) Conservation of 29 Sarbecovirus spike proteins with regions indicated as NTD (N-terminal domain, orange), RBD (receptor-binding domain, orange), RBM (receptor-binding motif, dark orange), S1/S2 and S2' (protease cleavage sites, black arrows), FP (fusion peptide, blue) and HR1/2 (heptad repeat 1/2, dark blue). Positions of mutations of selected bat Sarbecoviruses (gray) and SARS-CoV-2 VOCs (red) are indicated as circles. (**B**) Overview of the SARS-CoV-2 Spike structure (PDB: 7KNB) and localization of amino acid changes in the indicated SARS-CoV-2 VOCs. Color coding according to domains as indicated.

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The SARS-CoV-2 Spike protein is characterized by two proteolytic cleavage sites: the S1/S2 and the S2' site (Figure 2), which may have played a significant role in SARS-CoV-2 transmission and evolution[33]. The S1/S2 cleavage site is located at the boundary of the S1/S2 subunits of the Spike protein and distinguishes SARS-CoV-2 from related animal coronaviruses[35,36]. This polybasic site comprises an insertion (680SPRRAR↓SV687), forming a cleavage motif (RxxR) for furin-like enzymes, enabling proteolytic activation of the Spike protein promoting entry into host cells[37]. The polybasic site allows Spike cleavage during virus packaging, thereby significantly enhancing viral transmissibility and expanding its tissue tropism[36]. The furin cleavage site enhances the ability of SARS-CoV-2 to infect certain cell types and induce cell-cell fusion, which may promote efficient viral spread[38]. Thus, it is thought that this site played a key role in the rapid spread of the COVID-19 pandemic. The origin of the S1/S2 furin cleavage site in SARS-CoV-2 Spike, particularly the question whether it was present before or after zoonotic spillover, has raised significant interest and is still under debate[39–41]. Some animal coronaviruses carrying a polybasic furin cleavage site in their Spike protein have been reported[42]. However, furin cleavage sites are not present in pangolin or bat coronaviruses that are closely related to SARS-CoV-2[40]. Thus, it has been suggested that this specific cleavage site developed early in the process of the virus adapting to its human host[40], although the possibility that it predisposed an animal virus for efficient zoonotic transmission cannot be excluded[43]. Several SARS-CoV-2 variants of concern (VOCs) manifested mutations near the S1/S2 furin cleavage site, that altered Spike processing efficiency. For instance, the early Alpha and all subsequent VOCs acquired the P681H mutation increasing cleavage efficiency[44]. Additionally, the Omicron-specific N679K mutation hampered Spike processing and conferred a shift toward upper airway replication in hamster models[45].

The second step of proteolytic activation is mediated by S2' cleavage. It liberates the fusion peptide of the S2 subunit, enabling its insertion into the cellular membrane and subsequent formation of the 6-helix bundle mediating fusion of the viral envelope with the host cell membrane, a step crucial for viral entry into the host cell[46,47]. Two main types of host cell proteases, Transmembrane Serine Protease 2 (TMPRSS2) and Cathepsins, are involved in this process, and each plays a distinct role depending on the cellular entry pathway of the virus. When SARS-CoV-2 binds to the ACE2 receptor on the host cell surface, TMPRSS2 cleaves the spike protein at the S2' site[27]. In contrast, Cathepsins become involved when the virus enters cells via the endosomal pathway and fusion is triggered by the acidic environment of the endosome. Notably, it has been reported that the less efficient Spike cleavage of Omicron BA.1 Spike at S1/S2 is associated reduced dependency on TMPRSS2 and a shift towards Cathepsin-dependent endosomal entry. This shift in cellular tropism away from TMPRSS2-expressing cells is largely mediated by a H655Y substitution in Spike and may impact viral pathogenesis[48–50].

Both, the S1/S2 and S2’ cleavage sites of the Spike protein play a crucial role in the ability of SARS-CoV-2 to infect human cells and have been a focal point in understanding the virus's transmission dynamics and pathogenicity. The evolution of these sites in different VOCs may have significant implications for transmissibility, disease severity, and vaccine efficacy. Thus, continued monitoring and research into these mutations are essential for managing the pandemic and developing effective countermeasures.

In addition to using ACE2 and cellular protease, the Spike protein of SARS-CoV-2 has been shown to hijack usually antiviral IFITM2 and IFITM3 proteins for efficient infection[51–54]. Furthermore, it has been reported that the SARS-CoV-2 Spike counteracts the restriction factor tetherin that otherwise inhibits the release of viral particles[55]. However, if these mechanisms are conserved in the coronavirus family and to which extent they contributed to the efficient spread of SARS-CoV-2 remains to be determined.

Initial human adaptation of SARS-CoV-2 Spike proteins

Common cold coronaviruses, which circulate in the human population, are highly divergent from SARS-CoV-2. In contrast, SARS-CoV-1 Spike proteins share about 76% homology with that of SARS-CoV-2 and might induce cross-neutralizing antibodies[56]. However, SARS-CoV-1 infected only

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~8.000 individuals and has fortunately disappeared. Thus, SARS-CoV-2 essentially hit an immunologically naïve population and initial adaptations to humans manifested in changes that increased viral infectivity and transmission. For example, mutation of D614G in the receptor-binding domain (RBD) of the Spike became prevalent during the first few months of the pandemic, indicating a significant selective advantage for the spread of SARS-CoV-2 in human populations[57–60]. It has been reported that D614G alters Spike configuration, and enhances viral replication in human cells, as well as in the human respiratory tract, hence increasing transmission rates but not pathogenicity[59–63]. Another mutation that emerged relatively rapidly is N501Y thought to increase the binding affinity of the Spike protein to the ACE2 receptor[64,65]. Additionally, evolution of SARS-CoV-2 codon usage and the slower-than-expected acquisition of mutations, hinting at a purifying selection during the initial phase of the pandemic, provide insight into the virus's genomic adaptation strategies[66,67]. Alterations in the RBD of the Spike protein, which increased the affinity for the human ACE2 receptor were suggested to represent adaptive steps critical for efficient human-to-human transmission of SARS-CoV-2[68,69]. Since its emergence, thousands of mutations have been observed, with novel variations continuously emerging as the virus replicates and spreads across the human population[70].

Evasion of adaptive immunity

After SARS-CoV-2 infection and/or vaccination of significant parts of the human population, selection pressures shaping the evolution of Spike shifted from alterations increasing viral infection and replication fitness to mutations allowing evasion of humoral immune responses[19,21]. The initial variants of concern (VOCs), named Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Delta (B.1.617.2) all emerged independently. Each of these variants contained about 6 to 8 changes in the Spike protein, most of them in the RBD (Figure 2), promoting mainly immune evasion. However, changes affecting ACE2 binding and increasing fusogenicity have also been reported[21,71]. This has changed with the emergence of the Omicron VOCs that contained a strikingly high number of changes, especially in the viral Spike protein, compared to all previous SARS-CoV-2 variants[72]. The initial Omicron BA.1 VOC outcompeted the previously dominating Delta VOC at enormous speed, although it displayed low infectivity and replication fitness in many cell culture systems and animal models[49,73–75]. It was itself outcompeted by BA.2 which differs by ~20 amino acid changes in Spike from BA.1. All subsequent and current VOCs originated from BA.2 and contain further amino acid changes in their Spike proteins. Accumulating evidence suggests that initial mutations facilitated viral immune evasion at the cost of reduced infectivity and subsequent changes restored infectiousness and replication fitness[23,76]. New variants of Omicron are constantly emerging showing mutations in the RBD and the N-terminal domain (NTD) of Spike allowing immune evasion[77–79] (Figure 2) leading to the simultaneous emergence of sub-variants, each characterized by mutations that converge on several hotspots of their RBDs. Specific strains like BQ.1.1.10, BA.4.6.3, XBB, and CH.1.1 were identified as highly antibody-evasive[80,81]. This phenomenon of convergent evolution is driven in part by the humoral immune pressure, which promotes the evolution of the virus in a way that helps it evade nAbs[82]. Just like common cold coronaviruses, infection with one strain of SARS-CoV-2 does not efficiently protect against infection with another strain or newly emerging variants. For example, the XBB lineages that currently dominate the pandemic are largely resistant against neutralization by humoral immune responses induced by infection with earlier SARS-CoV-2 variants (including BA.1, BA.2 and BA4/5) or vaccination[83–86]. The SARS-CoV-2 variant of interest BA.2.86 that was first isolated in July 2023, has 36 amino acid substitutions compared to XBB.1.5, many of them located in key antigenic sites of the Spike protein. The proportion of BA.2.86 and closely related descendent lineages, such as JN.1 characterized by an additional substitution of L455S in the Spike protein, is currently steadily increasing indicating high transmission fitness and efficient humoral immune evasion[87]. However, T cell responses may remain effective and prevent severe disease in most cases[88,89]. Altogether, SARS-CoV-2 continues to infect humans but in relatively stable numbers and has transitioned from the pandemic to the endemic phase[90,91].

Broadly acting vaccines or therapeutics targeting the SARS-CoV-2 Spike protein

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The evolution of new SARS-CoV-2 variants also conferred resistance to therapeutic antibodies and vaccines. For example, neutralizing antibodies (nAbs) against SARS-CoV-2 initially inhibited a variety of virus strains and showed great promise in treating and preventing infections[92]. Initially developed nAbs target epitopes in the RBD that overlap the ACE2 receptor-binding site (RBS), thus sterically hindering binding of the Spike glycoprotein to its receptor. However, emerging SARS-CoV-2 variants show resistance to essentially all first generation FDA approved monoclonal antibodies (Figure 3)[93,94]. Specifically, mutations of E484A/K and Q493R in the RBD of the Spike protein rendered SARS-CoV-2 resistant to Bamlanivimab and N440K and G446S to Imdevimab[76,95]. The Omicron variants have shown an impressive capability to evade even those nAbs that target more conserved domains in the Spike RBD region[96,97]. However, while coronavirus Spike proteins are highly variable and tolerate numerous changes in their RBD some structural and functional features are highly conserved, offering perspectives for broad-spectrum antiviral agents.

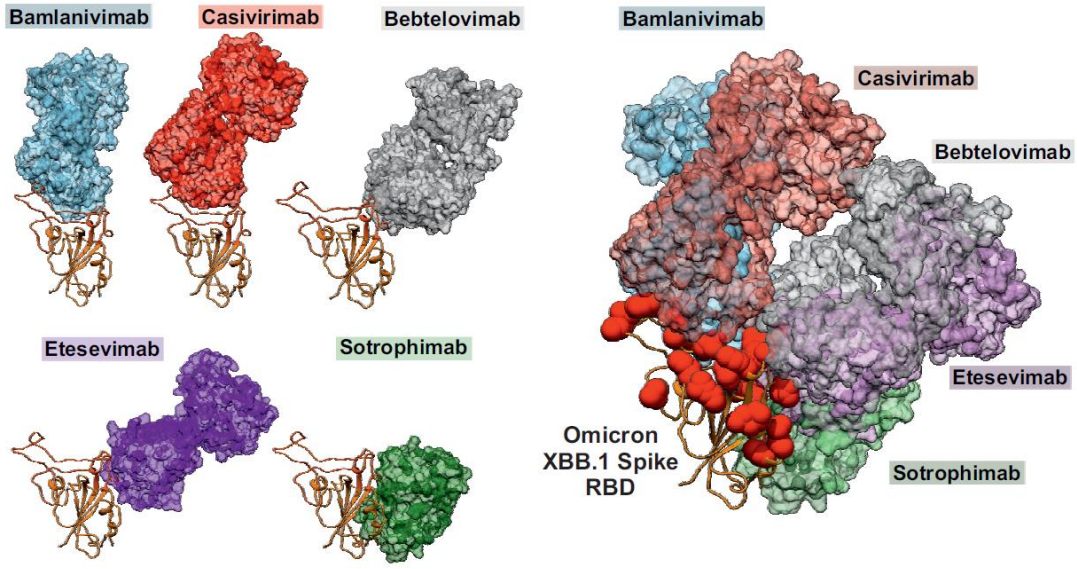

Figure 3. Illustration of binding sites of nAbs to the Spike RBD. The RBD of the SARS-CoV-2 Spike protein (PDB: 7KNB, yellow ribbon) and interacting therapeutic monoclonal antibodies are indicated: Bamlanivimab (blue, PDB: 7KMG), Casirivimab (red, PDB: 7M42), Bebtelovimab (gray, PDB: 7MMO), Etesevimab (purple, PDB: 7F7E) and Sotrovimab (green, PDB: 7TLY). The positions of RBD-specific mutations in SARS-CoV-2 Omicron XBB.1 are highlighted by red spheres.

Broadly neutralizing antibodies (bnAbs) designed to target highly conserved regions of the S2 region of the Spike protein including the fusion peptide and Stem-helix regions combine the potential for pan coronavirus activity with a high genetic barrier to evasion [98]. S2 stem helix binding bnAbs, isolated from SARS-CoV-2 recovered-vaccinated donors, showed broad cross protection against SARS-CoV-1, SARS-CoV-2, and MERS-CoV in mouse mdels[98]. Abs targeting the SARS-CoV-2 Spike fusion peptide not only reduced viral fusion, but additionally impaired proteolytic maturation of the Spike glycoprotein[99]. Thus, bnAbs targeting conserved domains in the S2 region of the viral Spike protein are promising candidates for next-generation pan-coronavirus vaccine development.

Peptide inhibitors are increasingly recognized in antiviral drug development due to their high specificity and biocompatibility. Similar to pan-coronavirus bnAbs, targeting conserved sequence and fusion mechanisms of the SARS-CoV-2 Spike protein S2 domain allows for the development of broadly, pan-coronavirus antiviral peptides. ACE2-mimicking, H1 derived peptides, are designed to mimic the key functional elements of the Spike protein - ACE2 interaction[100–102]. These peptides aim to competitively inhibit the binding of the virus to its ACE2 receptor, thereby blocking viral entry into host cells. An approach based on the principle of molecular mimicry, where the peptides structurally resemble the ACE2 interface that interacts with the spike protein of the virus[100]. Receptor usage by the SARS-CoV-2 VOCs is consistent, and although other receptors for SARS-CoV-2 have

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been identified, a switch in main receptor preference appears improbable. Thus, peptides mimicking ACE2 may effectively inhibit SARS-CoV-2 infectivity and offer a promising direction for developing long-lasting anti-SARS-CoV-2 treatments.

Just like many other enveloped viruses, such as HIV-1, entry of SARS-CoV-2 requires insertion of a fusion peptide into the cellular membrane and subsequent formation of a six-helix bundle between heptad repeat regions (HR1 and HR2) that pull the viral and cellular membranes together to mediate fusion. For instance, the peptide EK1 interacts with the highly conserved HR2 domain of the S2 subunit of the Spike protein (Figure 2), thereby preventing the interaction between HR1 and HR2 and hence virus-cell fusion[103]. Interestingly, EK1 and its optimized derivative (EK1C4) not only display broad or even pan activity against coronaviruses but even show cross-activity against HIV[104]. Mutation of N969K in Omicon Spike proteins induces substantial changes in the structure of the HR2 backbone in the HR1/HR2 post-fusion bundle. Nonetheless, EK1 and EK1C4 inhibit membrane fusion mediated by Spike proteins of Omicron subvariants[105]. In addition, inhibitors of the proteases activating coronavirus Spike proteins, i.e. furin, TMPRSS2 and Cathepsins may offer perspectives for broad-based inhibitors[106–108].

The initial vaccines developed against SARS-CoV-2 were designed based on the Hu-1 variant, the earliest strain of the virus[109–111]. These vaccines, including mRNA-based vaccines, like BNT162b2 (Pfizer-BioNTech) or mRNA-1273 (Moderna), vector-based vaccines like Vaxzevria (Astra Zeneka), or inactivated virus vaccines like CoronaVac (Sinovac) were highly effective in reducing COVID-19 pathogenicity and mortality[112,113]. In response to the highly immune evasive SARS-CoV-2 Omicron BA.4/BA.5 and XBB variants, adapted vaccines, like the bivalent Spikevax (Moderna) or the Omicron XBB1.5 adapted variants of BNT162b2, were designed to better match circulating strains[114]. Authorized for use, these vaccines aim to offer broader protection against COVID-19, preventing hospitalization and death due to infection. First studies demonstrate the enhanced effectiveness of these bivalent boosters against currently circulating SARS-CoV-2 variants compared to the original monovalent vaccinations[114]. Several strategies have been pursued to induce broad protection against diverse coronaviruses. For example, a trivalent sortase-conjugate nanoparticle vaccine that contained RBDs from SARS-CoV-2, RsSHC014 (a bat coronavirus), and MERS-CoV elicited neutralizing antibody responses against these viruses[115]. Another approach is based on mRNA vaccines designed to express chimeric Spike proteins aiming to elicit protection against a range of *Sarbecoviruses*, including SARS-CoV, SARS-CoV-2, and various bat coronaviruses[116]. However, neither previous coronavirus infections nor current vaccines confer long-term protection against all newly emerging SARS-CoV-2 variants. Thus, whether and how long-lasting and broad protection e.g. by alternative vaccination strategies can be achieved remains to be determined.

Conclusions and future perspectives

Coronaviruses have already been successfully transmitted from animals to humans on at least seven independent occasions. Furthermore, exposure to diverse *Sarbecoviruses* has been identified among high-risk human communities, providing epidemiological and immunological evidence that zoonotic spillover is continuously occurring[117]. Thus, broadly and long-lasting therapeutic and preventive agents are highly desirable. Human immune responses and newly emerging SARS-CoV-2 variants are in an arms race. Notably, even the most recent and divergent SARS-CoV-2 variants show only 3.3% (43 mutations in Omicron XBB1.5) to 3.5% (45 mutations in Omicron EG.5.1) amino acid diversity from the Spike proteins of the early virus strains. This is sufficient to confer efficient resistance against humoral immune responses induced by previous infection or vaccination. In comparison, bat coronaviruses and SARS-CoV-1 showing up to 30% amino acid diversity in Spike from SARS-CoV-2, are efficiently neutralized[31,56,118,119]. This illustrates the enormous power of the selection pressures in humans driving those changes in viral Spike proteins that allow efficient immune escape. On the positive side, this suggests that immunity induced by SARS-CoV-2 infections and vaccination might confer significant protection against newly emerging animal coronaviruses. However, it will be important to monitor if constant chasing of newly emerging SARS-CoV-2 variants by regular vaccine adaptation of may lead to immunogens showing increasing diversity from the

initial zoonotic pathogen. This raises the possibility that future vaccines could be effective against new SARS-CoV-2 variants but might lose activity against animal coronaviruses, thereby showing reduced potential in preventing future pandemics. Altogether, immunogens conferring broad protective responses are highly desirable and may also help to design effective preventive strategies against other viral pathogens.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

Author Contributions: F.Z. and F.K. wrote the manuscript. F.Z. and C.J. drafted the figures and F.K. modified and revised them. T.J. provided resources and supervised structural analyses.

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Conflicts of Interest: The authors have no conflict of interest to declare.

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