UJEMI PERSPECTIVES

Assembling an understanding: the critical role of SARS-CoV-2 envelope and membrane proteins in virion formation

Sara Kowalski

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY In March 2020, the World Health Organization declared coronavirus disease 2019 (COVID-19) a global pandemic. Since then, COVID-19 has claimed the lives of over 2 million people and is continuing to spread at an alarming rate, straining public health systems and crippling economies. The virus responsible for such global devastation is the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a *betacoronavirus* (ß-CoV) composed of a positive-sense, single stranded RNA genome surrounded by a viral envelope. The envelope of ß-CoVs consists of four major structural proteins: the envelope (E) protein, membrane (M) protein, spike (S) protein, and nucleocapsid (N) protein, with previous research implicating these proteins in the assembly and budding process of new virion particles. As these structural proteins are relatively conserved, previously acquired knowledge from other ß-CoV strains has helped characterize the structure and function of these viral envelope proteins in the context of SARS-CoV-2. However, our understanding remains incomplete surrounding the SARS-CoV-2 viral assembly and budding process and the host and viral proteins that are involved. This review will summarize the current knowledge on SARS-CoV-2 virion assembly and budding at the endoplasmic reticulum (ER)-to-Golgi intermediate compartment (ERGIC). Specifically, the structure of the major viral envelope proteins, E and M, and the conservation of their sequences will be discussed in detail, followed by a thorough examination of the functions of the E and M proteins during viral assembly at the ERGIC. Finally, an in-depth explanation of how these viral envelope proteins not only represent new target molecules for the development of novel therapeutics but also their potential as vaccine candidates will be highlighted. With the emergence of new variants exacerbating the state of the pandemic, advancing our understanding of viral and host proteins involved in the critical steps of the SARS-CoV-2 virus lifecycle remains a crucial aspect in continuing the fight against COVID-19. Expanding global knowledge on the molecular mechanisms of SARS-CoV-2 will provide further insight into the pathogenesis and epidemiology of SARS-CoV-2 infection and create new avenues for vaccine development and therapeutic interventions.

INTRODUCTION

The emergence of novel infectious diseases caused by zoonotic spillover events has increased over the past few decades and can be largely attributed to the growing interactions between humans and nature (1,2). Of importance are coronaviruses (CoVs), which possess high diversity and genomic variability making their 'jump' from animals to humans of great concern (3). *Coronaviridae* is a large and highly diverse family of enveloped RNA viruses, yet only two genera within the subfamily *Orthocoronavirinae*, alphacoronavirus and betacoronavirus (ß-CoV), are known to infect humans (4,5). Recognized as primarily infecting the respiratory and intestinal tracts of adults (6), human CoVs are among the many respiratory viruses that cause the common cold and are responsible for 10-30% of upper-respiratory tract infections resulting in mild disease (7). Nonetheless, as a consequence of pathogen spill over events, the emergence of three highly pathogenic CoVs, that are capable of causing severe illness, have entered the human population: severe acute

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Address correspondence to Sara Kowalski

saramkowalski64@gmail.com

respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and the cause for the current pandemic, SARS -CoV-2 (5).

The first known case of SARS-CoV-2 infection was detected in Wuhan China in December of 2019 and was found to cause the clinical disease COVID-19 (8). The symptoms of COVID-19 are very diverse ranging from mild disease presenting with a fever, cough, vomiting, and/or diarrhea to more severe disease manifestations resulting in severe pneumonia, acute respiratory distress syndrome, respiratory failure, or multiple organ failure (9). To date, the most common mode of SARS-CoV-2 transmission is through the inhalation of respiratory droplets and aerosols, with transmission due to fomites likely playing a minor role (10). Currently, there are no recommended, efficacious anti-viral therapies for the treatment of COVID-19, and the current vaccines, although effective against the original SARS-CoV-2 isolate, are showing decreased efficacy towards new variants such as the South African variant B.1.351 (11,12). Thus, a more comprehensive understanding of SARS-CoV-2 molecular biology is warranted to reveal additional avenues for drug therapies and vaccine candidates.

CoVs encode four major structural proteins in their genome: the envelope (E) protein, membrane protein (M), spike (S) protein, and nucleocapsid protein (N), all of which are multi-functional and involved in the production of new infectious viral particles (5). The SARS-CoV-2 S protein which creates the crown like spikes that distinguish CoVs, has received the most attention to date of the four major structural proteins. This is likely to due to its pivotal role during viral entry into susceptible cells via the angiotensin converting enzyme 2 (ACE2) receptor (13). However, this has caused an overlook of the remaining three structural proteins, in particular the E and M proteins, which control essential functions in multiple aspects of the viral lifecycle (14–16).

Assembly of new, infectious SARS-CoV-2 virus particles is a critical step during infection that occurs at the ERGIC inside host cells (17). The coordination of this process largely involves the multifunctional roles of the E and M protein. The E protein, once thought to have a minor role due to its size, is thought to not only play a part in membrane curvature and scission during budding, but also function as a viroporin in order to alter the host cell secretory pathway (18–20). These two contrasting roles may result from the E protein's adoption of two distinct (yet potentially simultaneous) structures during the viral lifecycle: a membrane helical (18) and transmembrane orientation (21). In contrast, the M protein is thought to be the main orchestrator of the assembly process due to its ability to interact with itself and other structural proteins (14,15). Similar to the E protein, the M protein can also assume two functionally different conformations (22). Given the critical functions of these viral proteins in the SARS-CoV-2 viral lifecycle, the M- and E-proteins may be both promising vaccine candidates and antiviral drug targets for COVID-19.

PROPOSED RESEARCH QUESTIONS

Although decades of research have been dedicated towards understanding the biology of the CoV lifecycle, the production of new virus particles and regulatory mechanisms governing viral assembly remain incompletely characterized. It is known that the E and M proteins have indispensable roles during viral assembly. Therefore, a comprehensive understanding on the structures of the coronaviral structural proteins, E and M, is integral in determining how they facilitate the assembly and budding processes and the production of infectious viral particles. Herein, I will discuss the structure and associated functions of the different E and M protein conformers during viral budding and assembly at the ERGIC and how they may present novel target molecules for the development of pharmaceuticals and vaccines against SARS-CoV-2. It should be recognized that due to the novelty of this CoV a relatively limited amount of knowledge pertaining to its molecular biology is available. Therefore, the information presented in this review will consist of research not only conducted on SARS-CoV-2 but other CoV strains.

PROPOSED PROJECT NARRATIVE

What are the structures of the SARS-CoV-2 viral envelope proteins E and M? Although the E protein is one of the smallest and least abundant constituents in the envelope of CoVs, it is an integral membrane protein that has been implicated in the retention of viral proteins at the site of assembly and the scission of budding virions. Ranging from 76 to 109 amino acids in length, the E protein is composed of three domains: a short hydrophilic N-terminus (~8 residues), an unusually long hydrophobic transmembrane domain (~25-30 residues), and a long hydrophilic C-terminal domain (~40 residues) (16) (Figure 1A). While a similar architecture has been observed in all studied CoV E proteins, including SARS-CoV-2, the primary sequence of the E protein is largely variant across the different CoV genera (<30% sequence identity). However, when assessing E protein sequence identity within the same genera, it is often highly conserved (23). Specifically, when comparing the primary sequence of the SARS-CoV-2 E protein to other ß-CoV E proteins from Pangolin and Bat CoV isolates, Bianchi *et al*. found the sequences to be nearly identical. The group also assessed the similarity of the SARS-CoV-2 E protein with the SARS-CoV E protein and discovered less yet still considerably high sequence identity (91% similarity) (24). The high structural conservation of CoV E proteins has been corroborated elsewhere (see Sarkar and Saha 2020), indicating the potential for highly conserved functionality of the SARS-CoV-2 E protein (Figure 1A).

The topology of transmembrane proteins is invaluable information when using structural data to predict protein function, underscoring the importance of determining the orientation of the CoV E-protein. With a variety of topologies being described in the literature (25–27), researchers have yet to come to a consensus on the membrane topology of the CoV E protein. Thus far, the only topology reported for the SARS-CoV-2 E protein has been that of an $N_{exo}C_{cyto}$ (28), which is able to oligomerize and form a viroporin in the lipid bilayer (similar to what has been documented for the SARS-CoV E-protein) (19,21,29). However, another contrasting topology has also been reported for the SARS-CoV E protein that is instead of a short palindromic transmembrane helical hairpin organization (18). Therefore, investigation into whether SARS-CoV-2 E protein also forms a helical hairpin conformation along with the stoichiometry that these two conformations exist at should be conducted. The functional significance of these two distinct topologies will be further explored later.

The M protein is the most abundant protein in the viral envelope and plays a central role in orchestrating the production of new infectious viral particles. The M protein is relatively large in size compared to the E protein (221-262 amino acids) and consists of five domains: a very short N-terminal domain, three transmembrane domains and a long C-terminus domain (Figure 1B) (30). As with the E-protein, the M protein sequence is reasonably well conserved across ß-CoVs. In the context of SARS-CoV-2, *Bianchi et al*. reported the SARS-CoV-2 M protein had a 98% sequence identity with Bat and Pangolin CoV M isolates, along with high yet relatively less identity to the SARS-CoV M protein (Figure 1B) (24). This high conservation highlights, as with the E protein, the potential for a conserved functionality of the M protein.

Unlike the E protein, there is less ambiguity surrounding the topology of the CoV M protein. An in-silico topology report of the SARS-CoV-2 M protein suggests that it adopts an $N_{exo}C_{cyto}$ orientation within the membrane, which is also reported for SARS-CoV and other CoV M-proteins (30–32). However, as the topology report is only a predictive model of protein structure, further in vitro studies using visualization techniques, like cryo-electron tomography or electron microscopy, should be conducted to confirm the SARS-CoV-2 M protein orientation. While there is general acceptance of the basic structure of the M protein, a lack of detailed structural information on the M protein during viral assembly remains. This lack of structural information is likely owed to the M proteins close association with the lipid envelope and its ability to form soluble aggregates making it difficult to isolate and study the M protein in its native conformation (22). As is common with many proteins, the M protein has been shown to be polymorphic, allowing for it to be multifunctional during the assembly process. Specifically, Neuman *et al.* discovered evidence that the M protein adopts two

distinct conformations at the site of viral assembly: an elongated M conformer and a compact M conformer. Although this has not yet been confirmed for the SARS-CoV-2 M protein, due to the conservation of the M protein, it is likely the SARS-CoV-2 M also adopts these conformations during infection.

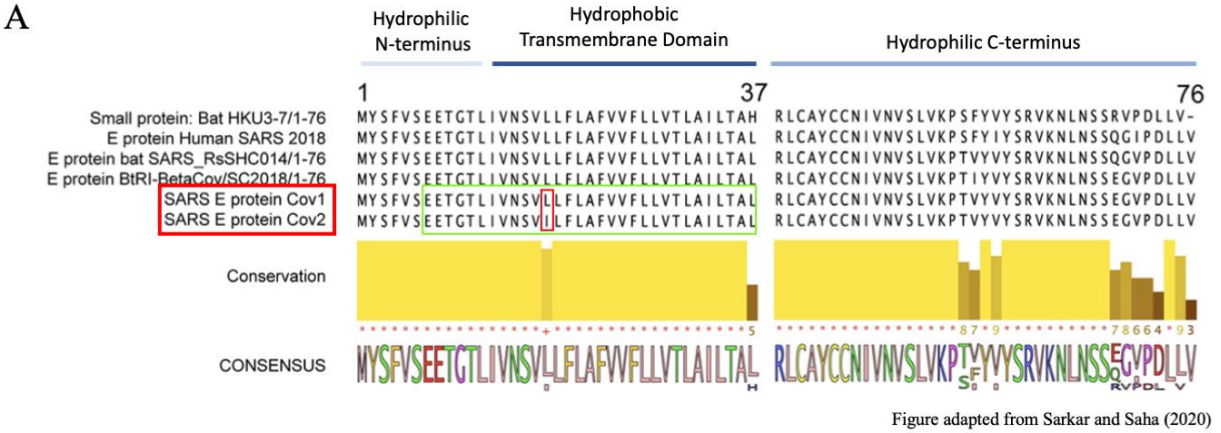

Figure adapted from Sarkar and Saha (2020)

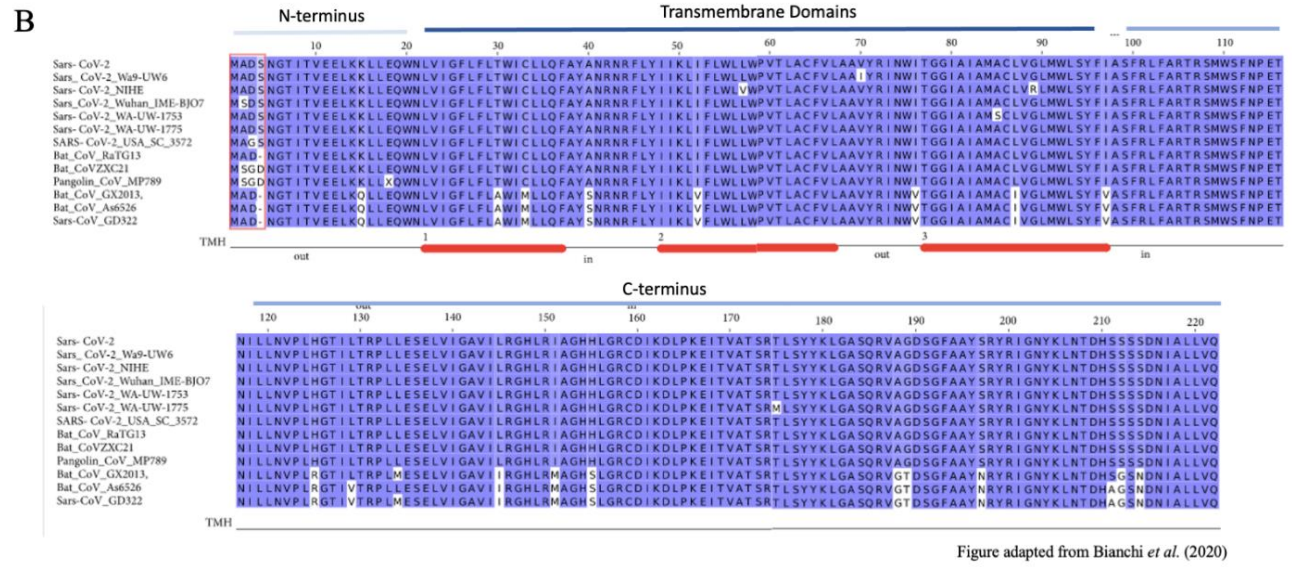

Figure adapted from Bianchi *et al.* (2020)

FIG. 1 Primary sequences of the SARS-CoV-2 envelope and membrane protein. A. Envelope protein sequences from Bat, SARS-CoV, and SARS-CoV-2 isolates. The green box represents the amino acids that compose the unique palindrome sequence found in the E protein. The red box represents the single amino acid difference between SARS-CoV and SARS-CoV-2 E protein sequences. B. Membrane protein sequences from SARS-CoV-2, Bat, Pangolin, and SARS-CoV isolates. Blue coloured amino acids denote conserved positions. Red bars under the sequences indicate predicted transmembrane helices. The red box represents highly variable sites at the N-terminus.

What are the functions of the E and M proteins during viral assembly and budding? Retention of the S protein in the ERGIC and the budding of new virions occurs, in part, due to the function of the E protein. As described above, the E protein has been demonstrated to assume two distinct conformations during infection: a helical hairpin and viroproin with an $N_{exo}C_{cyto}$ structural organization. Arbely *et al.* were the first to discover a novel pseudo-center of symmetry within the SARS-CoV E protein, which they suggest allows the E protein to form a helical hairpin within a lipid bilayer membrane. This same pseudo-center of symmetry is also found in the primary sequence of the SARS-CoV-2 E (28), suggesting that it too may adopt a helical hairpin conformation in the membrane. If true, this symmetrical structure

would implicate the E protein as playing a pivotal role in viral budding because the helical hairpin structure would assist in modifying lipid bilayers by increasing membrane curvature (18). In contrast, the proposed viroporin structure of the E protein would serve different functional purposes during the assembly process. Sarkar and Saha were the first to demonstrate that the SARS-CoV-2 E protein was capable of oligomerizing and forming a viroporin in the membrane using computational models (Figure 2A). They used the crystal structure of the SARS-CoV E protein to guide their model of the SARS-CoV-2 E protein which allowed them to identify potential key amino acids involved in the mechanism of the channel and construct water docking models to reveal dynamic conformational states of the E protein (Figure 2B)(19). The action of the E protein as a viroporin was further confirmed in separate invitro studies (20,33). Of particular interest, Boson *et al.* showed that the E protein is involved in retaining the S-protein and likely other viral components at the site of assembly by altering the host cell secretory pathway to slow down its maturation (20). Taken altogether, it is plausible that the CoV E protein assumes two distinct conformations during the viral lifecycle: a helical hairpin structure incorporated in small quantities into new virions to assist in membrane curvature and budding of the virus, and a viroporin with an $N_{exo}C_{cyto}$ topology that is responsible for altering the secretory pathway but is not incorporated into the envelope of the virus.

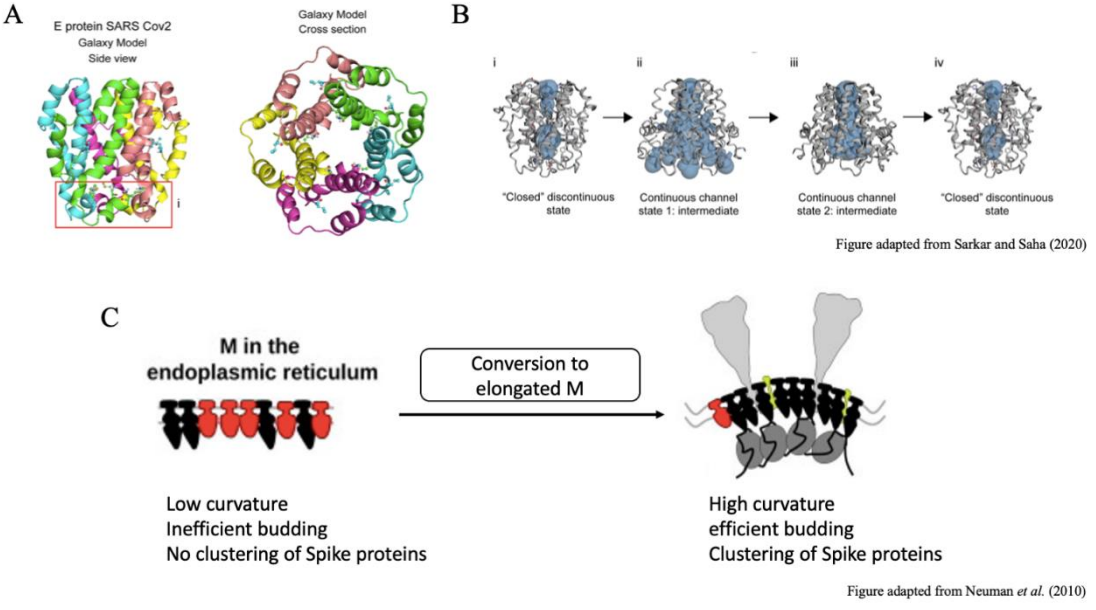

FIG. 2 Structural representation of the SARS-CoV-2 envelope and membrane proteins during viral assembly. A. Predicted viroporin model side and top view of the SARS-CoV-2 E protein. B. Predicted conformational changes of the E viroproin modeling the open and closed channels for the passage of ions. C. a modelled mechanism of the switch between compact M and elongated M to promote viral particle assembly. The red proteins represent compact M, the black proteins represent elongated M, and the grey protrusions represent S proteins.

The accumulation of viral components at the site of virion assembly is necessary for the production of new infectious viral particles. In CoVs this role is mainly carried out by the M protein. The M protein can interact with all of the major structural proteins of CoVs, including itself, in order to facilitate the packaging of the components into new virions (15). Specifically, the cytoplasmic domain has been shown to be indispensable for binding to the N protein to incorporate the genome into the new virion (14,15), while multiple domains of the M protein enable interactions with the E and S proteins in order to retain the S protein at the site of assembly and ensure the E protein is present for viral budding and scission (15). Thus far, in SARS-CoV-2, the only confirmed interaction has been that of the M and S protein. *Boson et al.* provide evidence for an interaction between the M and S protein through the cytoplasmic tail of the S protein that results in S protein retention at the site of assembly. Although interactions between the M protein and other viral proteins have not yet been investigated for SARS-CoV-2, due to the high structural conservation of the M protein, it is likely that the functional characteristics previously described for other CoV M proteins are

conserved. Still, investigation of these interactions should be conducted to assess if any of the interactions have been significantly altered as a result of specific amino acid changes in previously identified domains crucial for M protein function (34). As discussed earlier, there is evidence for the M protein to adopt two distinct conformers during the assembly process: a M compact and M elongated conformation, which have separate functional roles (Figure 2C)(22). In their analysis, Neuamn *et al.* discussed a mechanism of conversion between the two conformers to promote viral assembly, from M compact, which was shown to be associated with membrane flexibility and decreased S clustering, to M elongated, which was associated with clusters of S proteins and membrane rigidity and curvature (Figure2C)(22). Conformation of these M protein conformers in SARS-CoV-2, and further elucidation into their structural and functional characteristics should be explored to increase our understanding of the viral assembly process and in particular, the mechanism behind M conformer switching.

A

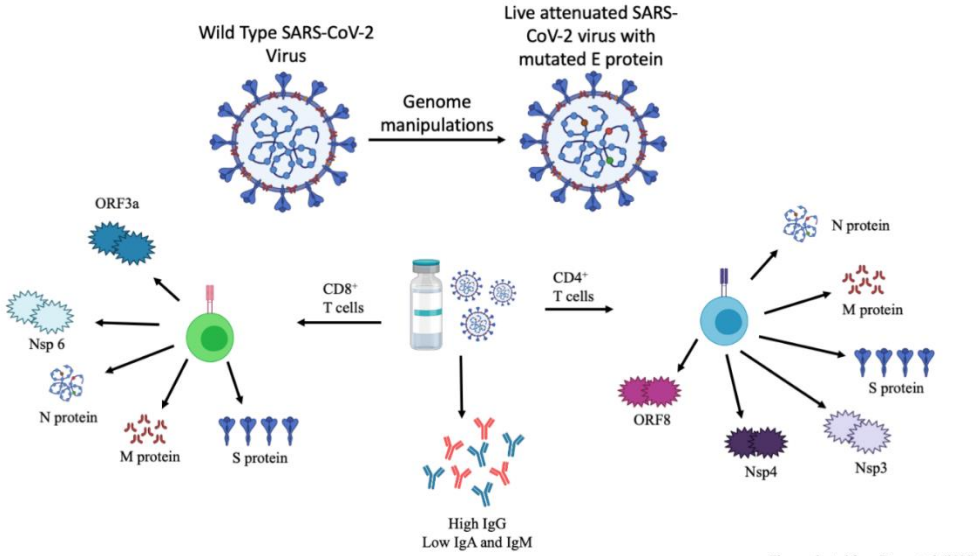

Figure adapted from Dong *et al.* (2020)

FIG. 3 Immune responses induced by a SARS-CoV-2 live attenuated vaccine. Live wild type virus is attenuated using laboratory techniques. Attenuated virus particles are then used for vaccination to elicit a broad range of responses which include both cellular and humoral immune responses. $CD4^+$ T cell responses are directed against M, S, N, Nsp3, Nsp4, and ORF8 proteins, whereas $CD8^+$ T cell responses are directed against M, S, N, Nsp6, and ORF3a proteins. The humoral response primarily generates IgG antibodies with low levels of IgA and IgM.

Are the E and M proteins good vaccine candidates and/or targets for anti-viral drug development? The most effective measure to stop viral spread and protect vulnerable populations from severe viral infection is the implementation of vaccines (35). However, due to the swift appearance and novelty of SARS-CoV-2, there is relatively limited data on newly developed vaccines and their efficacy, especially concerning their effectiveness on emerging SARS-CoV-2 variants. Currently, all the vaccines that are in use are designed to immunize against the S protein. However, there are other protein candidates worth considering that may be beneficial for designing live attenuated vaccines because they tend to have lower mutation rates compared to the S protein (19,36). Previously, the E protein was a promising candidate for designing a live attenuated vaccine against SARS-CoV (37–39). There is ample evidence that mutating the SARS-CoV E protein dramatically decreases viral titers compared to wildtype (WT) SARS-CoV, reduces inflammation, lung damage, and edema, and protects against lethal disease in immunized mice (37–40). Further, Sarkar and Saha demonstrated that the E protein, in comparison to the S protein, has a 50% lower chance of mutability, exemplifying its value as a vaccine and drug candidate. However, the potential for reversion of the vaccine is of large concern, and therefore, research assessing the safety of E protein vaccines need to be performed before human clinical trials can be considered (35). In comparison, studies on the M protein as a vaccine candidate have been limited. There is evidence that infection with SARS-CoV-2 causes the production of antibodies and triggers T cell responses directed against the M protein (Figure 3) (36,41), but there is a lack of information about the M protein's ability to be used as a target antigen.

In spite of there being multiple vaccines approved for emergency authorization against SARS-CoV-2, an efficacious drug regimen shown to treat infection still remains to be discovered. Studies looking into the repurposing of drugs as a fast and effective approach to treating COVID-19, have found multiple candidates, including compounds such as Gliclazide and Memantine which are two ion channel blockers that show potent inhibition of E protein activity in vitro (33). As Gliclazide and Memantine have already been approved for the use in treating type 2 diabetes (42) and dementia (43), respectively, they are clearly safe for use in humans; thus, clinical trials and safety and efficacy studies can move at a faster pace to assess their potential as SARS-CoV-2 antivirals. Another approach to treating SARS-CoV-2 infection would be through the design of novel antivirals amid at targeting specific SARS-CoV-2 viral proteins. Sarkar and Saha discovered key amino acids within the E viroporin that facilitate ion channeling action and allow the switch between open and closed conformers, highlighting the potential of small molecules designed to inhibit their function. This would prevent the alteration of the secretory pathway and the retention of viral proteins necessary for the assembly of new virions (20). The M protein is also a promising target for the development of novel antivirals. Studies show that specific domains of the M protein are necessary for its interaction with other structural proteins (15). Inhibiting these interactions would prevent the incorporation of essential viral components like the genome or the S protein which would ultimately prevent the production of infectious viral particles. There are a plethora of antivirals targeting the replication, attachment, and exit of the SARS-CoV-2 virus, with little attention on targeting the assembly of the virus (44). By developing small molecules to inhibit the E and M protein, multi-drug regimens can be developed that target multiple aspects of the viral lifecycle which would prevent emergence of resistant variants and increase antiviral potency (45).

CONCLUSIONS

The emergence of SARS-CoV-2 has led to an unprecedented global effort to develop vaccines and drug therapies in order to stop the widespread threat of this virus. However, a lack of complete understanding remains surrounding the viral assembly and budding process, specifically concerning the roles of the E and M protein. It is clear there is still ambiguity surrounding the structure and therefore the function of these proteins during viral assembly with multiple studies reporting different conformations of the E and M protein (22,46). Research into the effects of amino acid variations between CoV species and how they may impact the functions of these proteins should be conducted (Figure 1) (24). As well, further insight into the function of the E protein as a viroporin could come from other viruses that are known to encode viroporins in their genome such as Influenza and hepatitis C virus (HCV) (16). Studies looking into whether the HCV p7 or Influenza matrix-2 (M2) viroporins can replace the E protein during infection would provide insight into the conserved functionality of viroporins and important interactions occurring between the E protein and other viral proteins (Figure 2). Additionally, when developing novel drugs, researchers should aim to develop a broad-spectrum antiviral that targets not just the SARS-CoV-2 E viroporin but other virus viroporins so that it may be used for treatment against a wide array of pathogens. Finally, a detailed examination into the mechanism behind M conformer switching could guide the development of molecules to prevent this switch from occurring which would ultimately inhibit the formation of new infectious viral particles (Figure 2) (22).

The way out of this pandemic is through herd immunity, and the fastest way to achieve that is with vaccinations. Live attenuated vaccines are one of the most effective vaccine platforms to provide robust, broad, long-term immunity against pathogens. They more closely mimic a natural viral infection and therefore have the capacity to stimulate both the humoral and cellular immune responses against a broader range of antigens (Figure 3) (35). It has been well documented that knocking out or mutating the E protein can produce a safe and effective live attenuated vaccine (38,40). Further, currently approved vaccines are limited in their capacity to broadly stimulate the immune system as they are designed towards immunizing

against just the SARS-CoV-2 spike. Research has shown that those who are infected naturally with COVID-19 display an immune response against a wide array of antigens (36). Therefore, it is in our interests to start designing vaccines that elicit a broad array of responses as it may provide longer immunity and better protection against variants (Figure 3). However, with live attenuated vaccines there is the potential of reversion which raises safety concerns (35). One approach for circumventing this possibility is through the knockout or mutation of more than one viral gene in the genome. Knocking out multiple viral genes would essentially prevent the virus from reverting back as multiple mutations or burdens are harder to overcome as compared to one mutation (35). Given the benefits of a live attenuated vaccine on stimulating the immune system and the promising results from pre-liminary studies with a knockout E protein in SARS-CoV, further research should expand on these preliminary results and explore the possibility of a live attenuated vaccine for SARS-CoV-2.

It is evident from our experience of the current pandemic that we need to proactively prepare for future pathogen spill over events. This is not the first time a novel CoV has emerged, and it certainly will not be the last (1,5). In the interest of public health and preventing another CoV pandemic, scientists need to prioritize understanding CoV molecular biology in greater detail in order to develop broad-spectrum multi-drug regimens and produce effective annual CoV vaccines.

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