De novo design of anti-variant COVID vaccine with T-cell memory

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Abstract:

Recent studies have shown the efficacy of hybrid COVID-19 vaccines using wild-type nucleocapsid (N) and Spike (S) protein. We upgrade this strategy by one step further using clinically proven spike protein (but with delta and post-delta omicron mutations) and nucleocapsid peptides conferring T-cell immunity. As per the latest research nucleocapsid peptides are perfect immunological replacement of nucleocapsid protein. Therefore, peptide linking strategy is pursued (economic for cellular biosynthesis than whole protein). One envelope peptide with potent T-cell response is also chosen. This peptide is also functionally indispensable for the virus. All these peptides were clustered in our designed cytoplasmic domain separated by non-immunogenic helical linkers. We also propose the idea of introduction of any T-cell peptide similar to other Human Corona Viruses (HuCoV) in these linker regions whenever required. In addition to COVID, the same approach can be applied for any emergency or even long-term unsolved outbreaks of Influenza, Dengue and West Nile Virus etc. In this era of novelty as presented by subunit and nucleic acid vaccines, multiepitope strategies like this can help to combat multiple diseases successfully in real time to give hope for better future.

Introduction:

COVID-19 has brought the world to an unprecedented standstill since last four years (1). The massacre and chaos created are prevailing still. As this infection demonstrates wide clinical spectrum, from asymptomatic, mild, moderate disease to even long tern positivity and mortality (2). Also, new variants are appearing regularly with mutations in anti-spike antibody binding sites (3) causing new panic. Although, mortality in infected patients is lower than earlier SARS, serious further complications are seen in reinfected and even in vaccinated populations. These include myocarditis, damage to CNS etc that too in 30-40 years' population (4, 5). Reinfection with emerging newer variants is also a cause of concern (6, 7).

In this scenario question arises on sustainability of vaccines based on only wild type spike. Spike protein which generates neutralizing antibodies (8) is essential of course for recovery. But problem is direct mucosal immune response is short term. Long-term memory in mucosal immune response is generated by T cell response (8, 9). In children older than 2 years, infections by related corona viruses (Common cold) generate mild and asymptotic response against OC43, HKU1, 229E, NL63 (HuCoV) (10). Several immune response studies on HuCoV infections have been carried out (10, 11, 12, 13) so far. By the age of 2-5 years, 80% children are immunized and have persistent neutralizing antibodies (12).

Even COVID-19 infection demonstrated that T cell response is important to generate memory response and lower severity in infected patients (14, 15, 16, 17). Other studies concluded that nucleocapsid protein has to be included in further vaccine strategy (18, 19, 20, 21). This can be a promising strategy to curb circulating Delta (22), Omicron variants (23) and hybrids (24) simultaneously. Therefore, it is

important to design a vaccine with delta-omicron spike (22, 23) protein and T cell epitopes (Majorly from patient studies in literature). For this, we have designed a construct using chimeric COVID spike and COVID T cell epitope sequences. Structural proteins with almost conserved sequences for different corona virus families were targeted for potential T-cell enticing candidates. Functional proteins were not considered since some of them are known to hijack MHC systems in human (25, 26, 27). There are numerous studies now for homology of nucleocapsid protein sequences between COVID and HuCoV strains (18, 28, 29, 30, 31). Also, T-cell response of COVID unexposed individuals to COVID was observed for at least one peptide from envelope protein (32) indicating potential effect from common cold immunity. Therefore, we exclusively focused on nucleocapsid protein and envelope protein for T cell epitopes and compared with convalescent patients' T cell response inducing sequences from literature. Also, more attention was given on CD4+ TCR epitopes than CD8+. Since the former sequences were found to be more stable than the other as per studies on patients (33). Finally, the designed sequence had 5 epitopes joined by linkers as cytoplasmic region. Among these, at least two epitopes were found folded after model building for the sequence without trans-membrane domain. As more well-formed structural epitopes can ensure more CD4+ T-cell activity (34) to cause persistent immunity. Thus we propose that this sequence should be considered for final vaccine construct against COVID and any other future variants/viruses. Because, the linker regions can also be used as potential sites for insertion of epitopes against new viruses in future.

Methods:

Clustal Omega analyses of SARS-1 and SARS-2 spike sequences and B-cell epitopes prediction: Clustal O alignment (35) of SARS-1 and SARS-2 (COVID) spike native sequences (from Uniprot) (36) was done using EBI server. The sequences were further subjected to B-cell epitope prediction in IEDB server (37). The predictions were based on Kolaskar method, surface accessibility and a hybrid mode of both. The predicted B-cell epitopes were colour coded according to the method used (Fig. 4). The individual protein domains were also mapped on the aligned sequences using information from both clustal alignment and Uniprot. The amino acid mutations corresponding to Delta and Omicron spikes were also taken from Uniprot. All these mutations are substituted in native COVID spike sequence to design hybrid spike. The native furin site (PRRAR) is kept since it is a hotspot for immune response (from Uniprot).

Selection of peptides from literature: T cell epitopes from COVID recovered convalescent patients' studies were selected from available vast literatures (14, 15, 17, 28, 29, 30, 32, 38). The search was more for CD4+ epitopes as it was found CD8+ epitopes were heavily mutated (causing nonpersistent immunity) in different patients' samples (33). We mostly selected potent epitopes with definite secondary structures as per PDBsum. Nucleocapsid protein peptides and envelope protein peptides were

given most importance including one memory peptide from the former (with both T-cell and B-cell inducing capabilities). The similarities with homologous proteins from common cold coronavirus families as per literature studies were given utmost importance. Peptides highly similar to human proteins and closely situated in neighbouring regions in individual open reading frames (ORFs) are excluded from our selection to avoid allergenicity (39). Finally, the secondary structures of the selected peptides are verified from PDBsum (40) or Phyre2 (41) (in absence of PDB structure) analysis to prevent misfolding in the design.

Sequence conservation studies on Corona spike, Nucleocapsid, Envelope and Membrane proteins: Clustal omega sequence alignments for these proteins were generated using Uniprot data on COVID, SARS and common cold coronavirus. Further, the alignment were refined by removing gapped sequences, non-human host harbouring coronavirus homologs and partial protein fragments. The final alignment and associated sequence similarity based neighbour joining tree (without distance corrections) were generated with maximum 5 iterations as possible in Clustal O. The guide tree was plotted in iTOL (interactive Tree of Life, ver. 6.5.8, EMBL) (42).

Design of vaccine sequence: The hybrid sequence had mutated amino acids from both Delta and Omicron spikes. The core β strand was deleted from S2 region to prevent pre-to post fusion transition as shown before for coronavirus spike transitions (Fig. 6, 43). Potential Antibody dependent cellular cytotoxicity/Antibody dependent enhancement (ADCC/ADE) region (44, 45, 46) mapped from Clustal O (Fig. 4) was further fine-tuned by structural alignment with SARS spike in PyMOL (Fig. 5). The minimum region destabilizing the same was deleted. The cytoplasmic domain was engineered with careful placement of N-epitopes interconnected by non-immunogenic EAAAK linker (47, 48, 49). These epitopes were kept according to their positions in native nucleocapsid. Last two N-epitopes actually interact with each other in PDB structure (Supplementary Fig. S6). Therefore, envelope peptide (15 residues) is placed between linkers among them to help in interaction (to cancel zero neighbour interaction effect from rigid EAAAK linker helices).

***de novo* model building:** The resulting sequence is first modelled in Robetta server (RoseTTAFold, BakerLab) using default settings (50). Further, the model buiding was done in AlphaFold in Uppsala server (UPPMAX, Uppsala Multidisciplinary Center for Advanced Computational Science, Uppsala University, Sweden) (51, 52). The analyses were done in alpha-viewer python package (Severin Dicks, IBSM Freiburg) and PyMOL (The PyMOL Molecular Graphics System, Open-Source version, Schrödinger, LLC). In parallel, models for hybrid spike without transmembrane domain and wild type COVID spike were also made by AlphaFold. For comparison, all three models were also made by monomer pTM (predicated template modelling) mode to obtain PAE (predicated aligned error) map. Finally, predicted local distance difference test plots (pLDDT) were calculated for all models.

Results:

Uniprot analyses:

Uniprot mining of COVID strcutural proteins (spike, nucleocapsid, membrane and envelope protein) were divided into 3 parts: a) Retrieval of COVID, SARS, common cold and MERS sequences, b) Clustal Omega alignment of each structural protein from Uniprot and c) Sequence similarity based neighbouring-joining tree generation (without distance values) in Clustal Omega. At first, sequences corresponding to coronaviruses infecting all host animals were considered. For nucleocapsid sequences, we found out inclusion of MERS nucleocapsid generates a distinct tree which slowly diverges from other corona including COVID (Fig 2A). Further, we generated circular tree by filtering out gapped sequences, protein fragments and non-human host infecting corona viruses. This confirmed that MERS nucleocapsid protein sequences indeed form a distinct tree, but by being more conserved as compared to others (Fig 2B). In fact, it did not even diverge much within MERS family (Fig. 2B). Usually, nucleocapsid protein is highly conserved than spike (53) and more immunogenic (53, 54). It is also essential for maintaining RNA structure, packaging etc inside viral particle (55). If random mutations arise in nucleocapsid, virus will not survive in long run. Taking this MERS scenario, introduction of recurrent random mutations in nucleocapsid protein (by immunological attack) may be beneficial for vaccine design. Although, COVID (SARS-2) nucleocapsid has more mutations than MERS, still the frequency is much low compared to spike (Fig. 2B, Supplementary Fig. S1). Alongside we also generated similar sequence-similarity trees with other structural proteins (Spike, Membrane and Envelope) (Supplementary Fig. S1, S2 and S3). The focus was on 2 conditions: 1) Conserved structural proteins among living corona viruses (MERS, COVID and common cold corona viruses; not SARS as it is already extinct by natural immunity); 2) How immunogenic these proteins are. There we found out that envelope protein also can be a suitable candidate like nucleocapsid. This protein sequences were highly conserved among COVID strains (Supplementary Fig. S3). Thus we have taken one non-allergenic (39) epitope from envelope protein which is important for binding to human cell junction proteins (56) and has robust immune response (32). Surprisingly, spike protein sequences were also found highly conserved among MERS (Supplementary Fig. S1). As already chosen in existing vaccine candidates including our design, not much study was done on this further. Membrane protein (M) was found highly conserved among extinct SARS strains (Supplementary Fig. S2) and immunologically less persistent (described below), therefore not chosen.

Literature hunting for potential T-cell epitopes for vaccine design:

Immunogenic sequences of COVID nucleocapsid protein and envelope protein were carefully shortlisted from literature studies. Initially, the focus were on the sequences with overlapping CD4+ and CD8+ epitopes. Similarity with common cold viral sequences were also taken into consideration. The data available from recovered patient and asymptomatic population were given highest importance. Final

chosen peptides were screened based on their structures (from PDBsum) or from secondary structure predicted by Phyre2 server (in absence of PDB for envelope protein; Supplementary Fig. S7). As known, SARS-2 CD8+ epitopes are hotspots of nonsynonymous mutations to cause short-lived immunity (33). Since CD8+ epitopes are mostly unstructured, special consideration was made to choose epitopes with definite structures to attract CD4 based immunity. Subsequently, two epitopes were found folded in Alpha Fold. For the rest, we found one epitope fully and another partially non-structural after validation in Alpha Fold (described below). They were kept in the final construct based on their ability to provoke instantaneous immune response due to high similarity with common cold (28, 29, 30) and strong memory response (18, 38). Membrane protein epitopes were found less persistent as per the literature study (32, 54) with exception of highly COVID-exposed hospital workers (57) who are more immune to COVID than any other population. The chosen peptides are described in Supplemental Table 1.

Model building in RoseTTAFold (Robetta server, Baker lab, HHMI) and further validation in AlphaFold (Deep Mind): The designed sequence had signal peptide, transmembrane domain and a cytoplasmic domain formed by T-cell epitopes interconnected by EAAAK linker. Major portion of ADCC/ADE region and core β-strand were removed. The positively charged Endoplasmic reticulum (ER) exit signal from COVID native spike was placed at the cytoplasmic end to facilitate easy exit from ER (58). This sequence was first modelled in RoseTTAFold and found to be structurally similar to native spike, all domains including cytoplasmic domain are distinctly well formed (Fig. 7). To validate further, we have modelled in Alpha Fold for two sequences: with and without transmembrane (ΔTM) region. It was seen the at least two epitopes (NFGDQELIRQGTDYKHWPQIAQFAPSASAFFGMSR and FYVYSRVKNLNSSRV) were folded in ΔTM sequence (Fig. 8). While first sequence is well folded in the TM+ (with transmembrane) construct, but not the other (Fig. 10). It appeared like that the first sequence is the major driver of folding for the designed cytoplasmic domain. Also inter-residual distance analysis in PyMOL showed almost double distance (16.2 Å) between this peptide and last sequence or memory epitope (QVILLNKHIDAYKTFPPTEPKKDKKKKADET) in TM+ construct than the other (Fig. 9). The other peptide (DLSPRWYFYYLGTGP) and memory epitope are fully unstructured in TM+ construct. Memory epitope is partially structured in ΔTM possibly due to closer distance (8.2 Å) (Fig. 11A) to folding driver peptide. Seemingly, this closeness is resulted from absence of transmembrane domain in ΔTM which otherwise pulled away (Fig. 9B) the cytoplasmic domain from main protein in TM+ model. In fact, both TM+ model and native spike (having transmembrane region) have shown unstructured cytoplasmic domain which is further validated by PAE scores similarities (Supplementary Fig. S9, S10) in this domain for these two models. For the main protein, the receptor binding doman (RBD) is found to be well folded and identical among all three models including native spike (Supplementary Fig. S4). Also, the core β-strand is replaced by two discontinuous stretches of β-strands which can form hydrogen bonds to the complementary strand in core β-sheet (Supplementary Fig. S5).

Thereby, the structural integrity of this region can be maintained. Although, the lengths of these strands are not enough to support pre- to post fusion transition of spike unlike native core β-strand.

Discussion:

This paper describes bioinformatics design of a molecule with potential to overcome COVID and any possible viral attack in future. Conserved structural proteins of corona viruses are targeted in our study. Functional proteins are avoided as some of them have reported adverse effects on immune system. Therefore, our vaccine constructs are designed with SARS-2 structural proteins' T-cell evoking epitopes screened from the literature. The hallmark of current COVID vaccination is COVID wild type spike protein because of its potent antigenicity. But presence of vaccine evading variants pushed us to add mutant spike in our design. In pre-vaccination era, there was deadly delta variant which was followed by milder omicron variants after mass vaccination. This massive paradigm shift predicts future variants will be more like these two and/or hybrids to evade vaccine generated immunity! Therefore, hybrid spike sequence with both delta and omicron mutations is used. Furthermore, spike protein's S2 segment has regions which do not generate neutralizing antibodies and do not have dominant T cell response. There is also a short cytoplasmic domain. We have replaced it by inserting COVID's T cell epitopes interconnected by non-immunogenic, helical & rigid linker sequence EAAAK (47, 48, 49). To incorporate all these, we also have removed a region known as core β-strand (buried deep inside spike core, structurally dispensable in pre-fusion spike and immunologically less significant) (Fig. 4, Fig. 6) from the S2 region of spike. This can help to add new segment to the whole designed molecule without making it too big. Additionally, the absence of this region can prevent the spike to go from pre- to post fusion conformation (Fig. 6). Thus, standard proline-proline (non-viral sequence) insertion which is used in other spike based vaccines (59) is not needed. We also have removed a putative region in S1 which caused ADE responses in SARS as per literature study and structural analysis (Fig. 5B). Although ADE response is diminished in current COVID strains, there is no warranty it will not resurge in incoming variants! Since there is already report of COVID vaccine induced ADE type symptoms (Vaccine-associated enhanced respiratory pathology) in hamsters (60)! Finally designed construct have two sequences: with and without transmembrane domain. Both are with engineered cytoplasmic regions replacing native domains.

For selection of T -epitopes to build cytoplasmic domain, non-allergenic peptides among nucleocapsid's numerous T cell epitopes (Fig. 3) were enlisted including memory peptide (18, 38) which helps strong immune responses in recovered and vaccinated patients (besides mouse models). These sequences are also highly similar to other common cold coronavirus (HuCoV) nucleocapsid protein sequences (Fig 1). As all humans have immune response against HuCoV from childhood driven by pulmonary immune memory including CD4+ epitopes. So using these epitopes has an advantage to generate long term and quick immune response (13, 21). This establishes their importance in persistent immunity. Next

questions were, will these peptides cause misfolding in the generated construct? Subsequent PDBsum analysis helped to screen nucleocapsid peptides which were well formed with definite secondary structures (Fig 3B). For envelope peptide without any PDB structure, we have relied on Phyre2 result (Supplementary Fig. S7). Later model building in RosettaFold showed these peptides as alpha-helical in the designed cytoplasmic domain of the construct. Final Alpha Fold model building predicted that at least two peptides are folded while the other two epitopes are mostly unstructured in construct without transmembrane region. One of the folded epitopes can be the main player in folding for other subsequent peptides. The predicted LDDT values were >70 for this epitope in both ΔTM and +TM sequences (Fig. 8, Fig. 10). Also, we found out that the transmembrane domain pulls away the cytoplasmic domain from the other portions possibly due to its hydrophobicity in +TM construct. The resulting distance between last epitope and folding driver epitope doubles in +TM construct than ΔTM construct. Interestingly, the folding driver peptide was constructed by joining numerous CD4+ and CD8+ responsive N-peptides from immunological studies (Supplemental Table 1). In native Nucleocapsid protein, they are located as flanked to each other, parsed by immune system as numerous small peptides. Apart from immunological significance, they might play a role in folding of native nucleocapsid around viral RNA. Further studies on this can be done to prove this hypothesis.

As conclusion, we propose that this ΔTM construct with linker joined cytoplasmic epitopes can be a potential vaccine candidate against deadly COVID or any other virus! As other than providing support for the structure and immunogenic framework, there is also a future perspective for the linkers used in our construct. We keep the window open for inclusion of new epitopes replacing the spacers and/or obsolete (if happens to be) epitopes used in our construct. Post-COVID world is experiencing a lot. From human infecting ability of several zoonotic viruses like monkeypox virus (61), sarbecoviruses (62), lumpy skin disease viruses (63) etc to rise of deadly viruses like dengue etc (64). All these pinpoints to the need of a combinatorial strategy for pre-priming the immune system to combat COVID like pandemic and new infections of exotic and existing viruses thereafter. Also, currently there is an abyssal void for the treatment options for the children below 2 years, since most focus for current vaccination strategies are from adolescence. All these can be rectified with a readily available vaccine construct for all age groups with a potential for inclusion of epitopes in emergency.

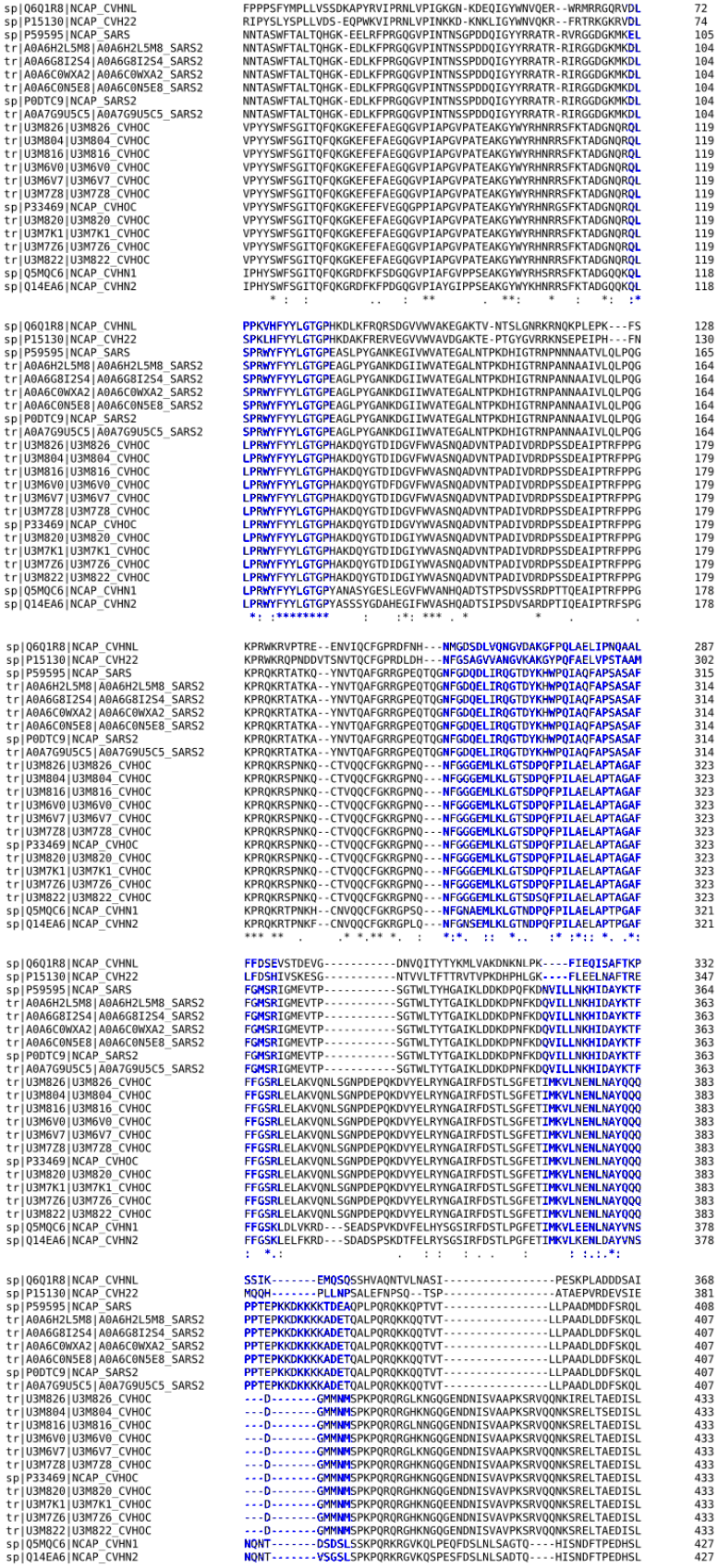
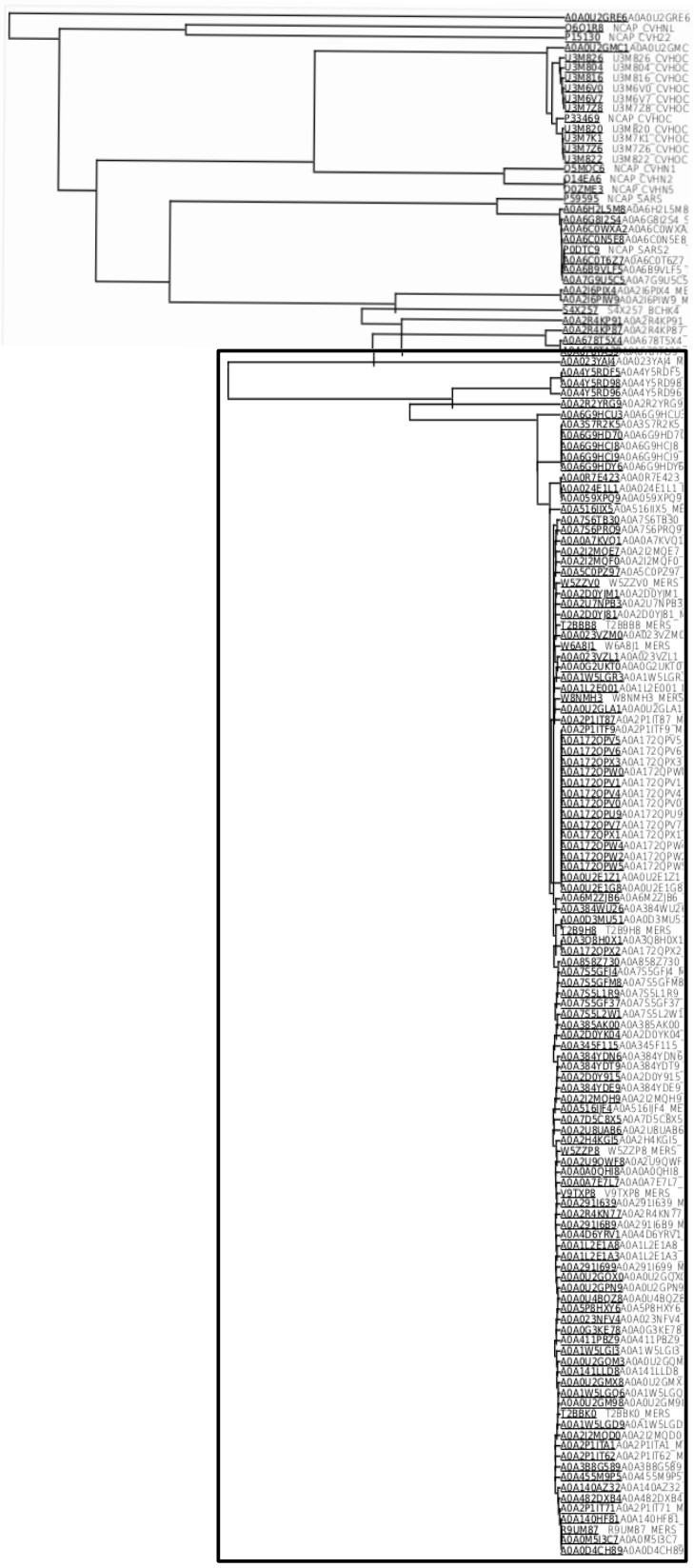


Fig 1: Clustal Omega alignment of Nucleocapsid (N) protein sequences. The sequence alignment among SARS, COVID and common cold coronaviruses shows high similarity in chosen epitopes (blue). Except last several residues in last peptide, all SARS2 N-epitopes are highly similar to HuCoV sequences.

A)



B)

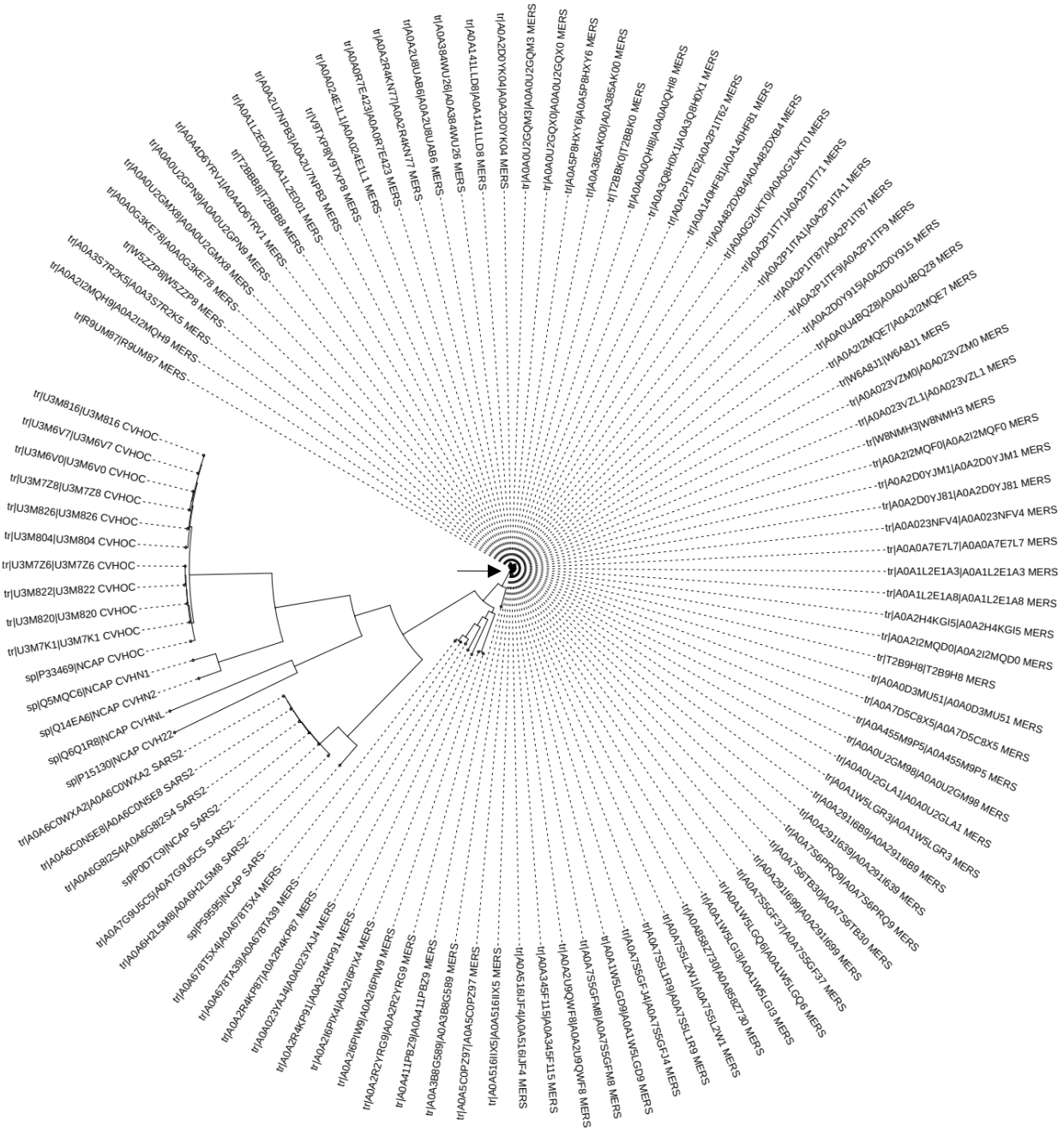


Fig 2: Sequence similarity-based guide tree of Coronavirus nucleocapsid sequences.

A) Preliminary guide tree from Clustal Omega alignment of nucleocapsid sequences. Uniprot-retrieved Corona virus N-protein sequences' alignment demonstrates distinct unique tree formation from later variants of MERS (box enclosed). Here all sequences as available from the database are used. **B)** Sequence similarity-based neighbouring-joining circular tree of coronavirus nucleocapsid proteins sequences. These are chosen from Uniprot after removing fragments and non-human host infecting coronaviruses. The circular tree is plotted in iTOL. Arrow marked are MERS nucleocapsid proteins showing very little changes in their sequences during as compared to others.

A)

>Nucleocapsid protein

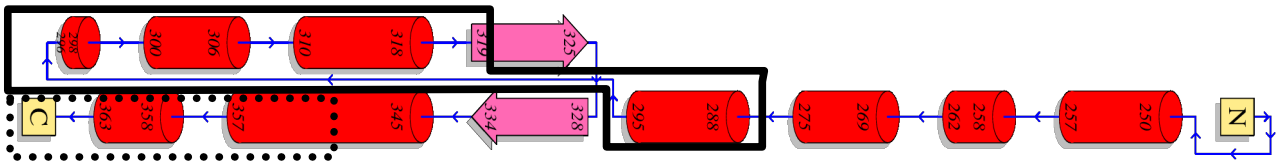
MSDNGPQNQRNAPRITFGGPSDSTGSNQNGERSGARSKQRRPQGLPNNTASWFTALTQHGKE
DLKFPRGQGVPINTNSSPDDQIGYYRRATRRIRGGDGKMK(103)**DLSPRWYFYYLGTGP**(117)
EAGLPYGANKDGIIWVATEGALNTPKDHIGTRNPANNAAIVLQLPQGTTLPKGFYAEGSRGGS
QASSRSSSRSRNSSRNSTPGSSRGTSPARMAGNGGDAALALLLLDRLNQLESKMSGKGQQQQ
GQTVTKKSAAEASKKPRQKRTATKAYNVTQAFGRRGPEQTQG(285)**NFGDQELIRQGTDYK**
HWPQIAQFAPSASAFFGMSR(319)IGMEVTPSGTWLTYTGAIKLDDKDPNFKD(349)**QVILLN**
KHIDAYKTFPPTEPKKDKKKKADET(379)QALPQRQKKQQTVTLLPAADLDDFSKQLQQSM
SSADSTQA

>Envelope protein

MYSFVSEETGTLIVNSVLLFLAFVVFLLVTLAILTALRLCAYCCNIVNVSLVKPS(56)**FYVYSRV**
KNLNSSRV(70)PDLLV

B)

I)



II)

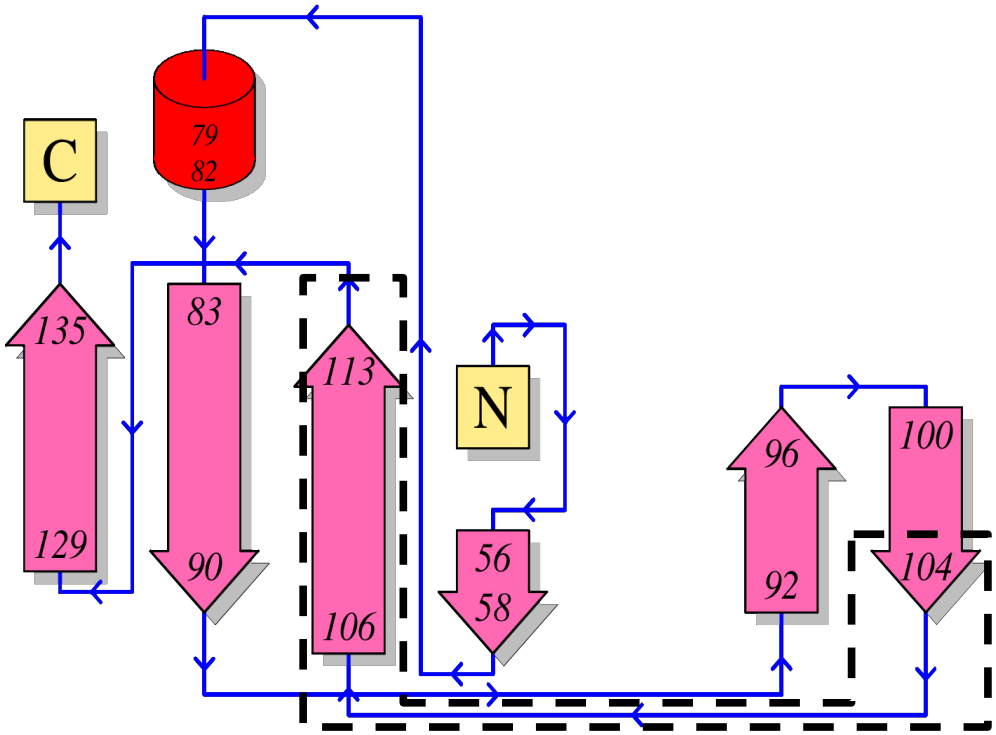
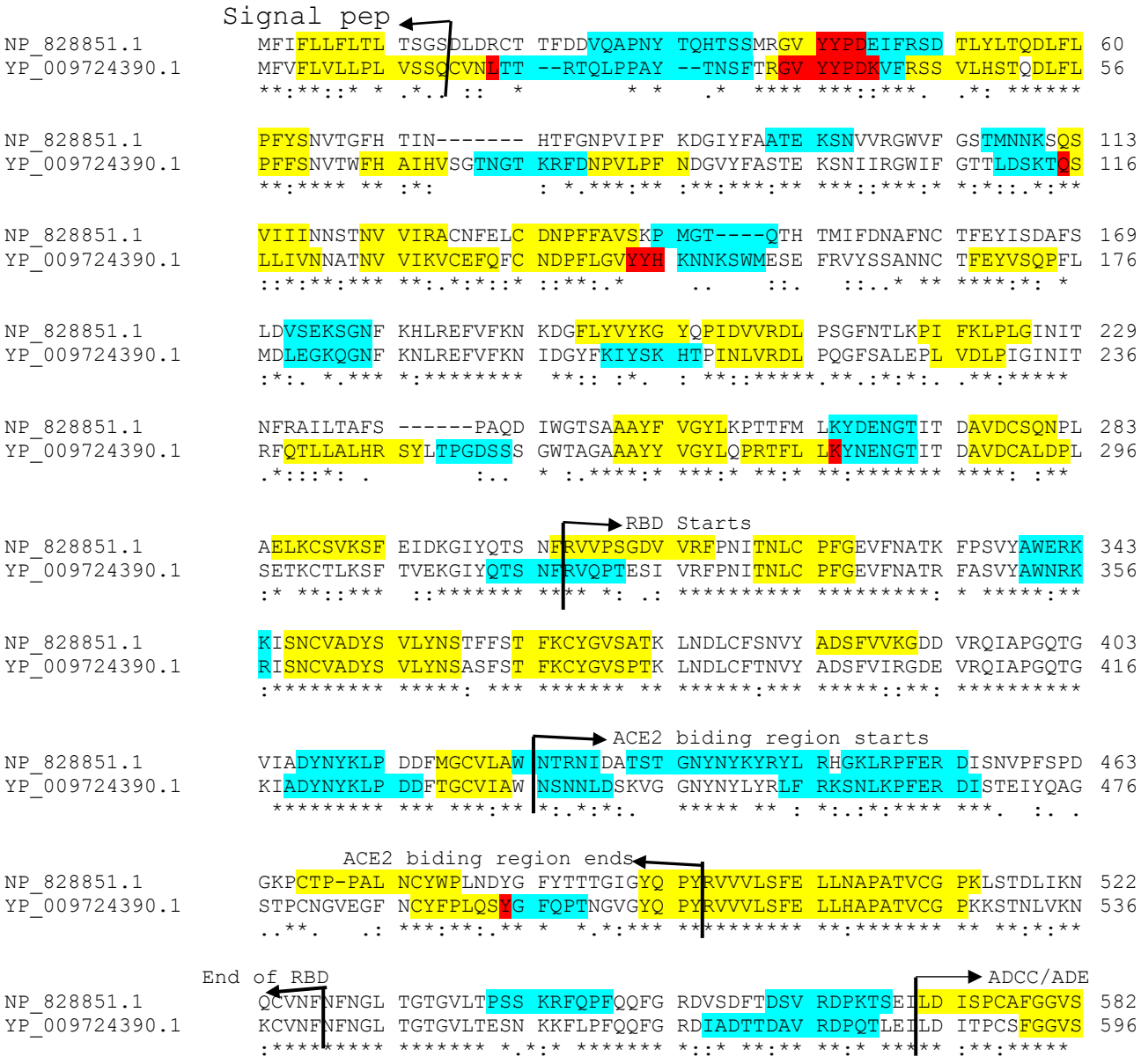


Fig 3: Wuhan COVID Nucleocapsid and Envelope protein epitopes.

A) The T-cell epitopes from N and E protein sequences (in FASTA format), homologous with common cold, chosen for the cytoplasmic domain design of the vaccine construct. These are highlighted yellow, bold-lettered and underlined. The numbers in parenthesis denote amino acid positions.

B) Secondary structures of the chosen nucleocapsid peptides in enclosures from PDBsum: I) **QVILLNKHIDAYKTFPPTEPKKDKKKKADET** (**6ZCO.pdb)** in dotted rectangle has mostly α-helical structure with adjoining loops; 15 residues missing at C terminus. **NFGDQELIRQGTDYKHWPQIAQFAPSASAFFGMSR (6ZCO.pdb)** in continuous lined enclosure has α-helical structures with adjoining loop. II) **DLSPRWYFYYLGTGP** (**7N0R.pdb)** in dashed enclosure has non-complementary β-strands along with adjoining long loop thereby strong possibility of forming unstructured epitope in construct design. The core sequence in this epitope is **SPRWYFYYL** which induces CD8 response (Supplemental Table 1). CD8+ epitopes are usually linear/unstructured.

CLUSTAL O(1.2.4) multiple sequence alignment



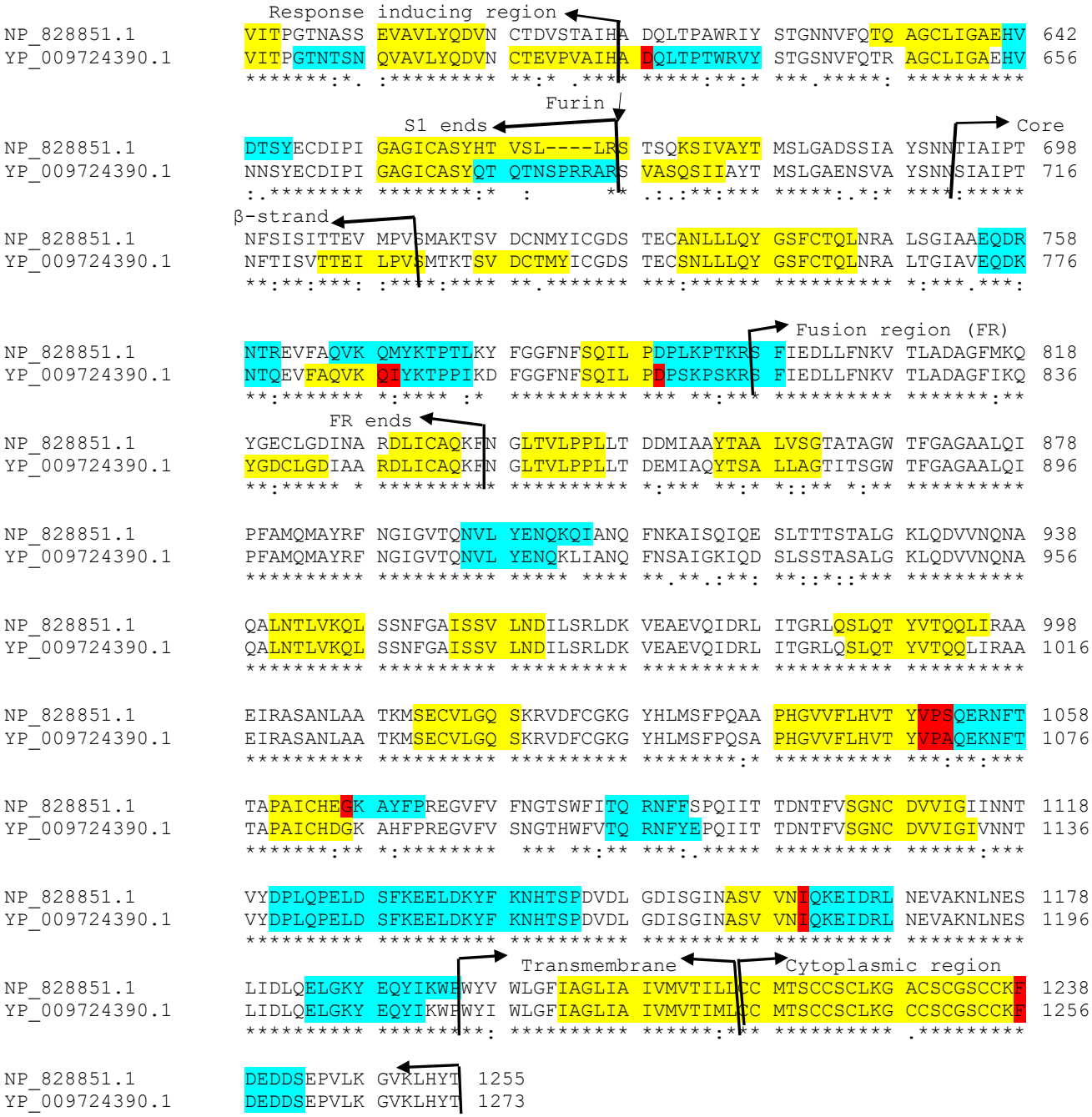


Fig 4: Mapping of B cell epitopes regions of Wuhan COVID spike (YP_009724390.1). Native SARS2 spike RBD, ACE2 binding sequence, furin site, fusion region along with ADCC/ADE inducing regions are aligned with SARS spike (NP_828851.1) in Clustal Omega. The B-cell epitope analysis is from IEDB server. Yellow is B cell epitopes by Kolaskar method, Cyan blue is surface accessibility. Red is having both methods. These B cell epitope regions are present in delta and omicron spike also with ever-changing sequences evading immunity.

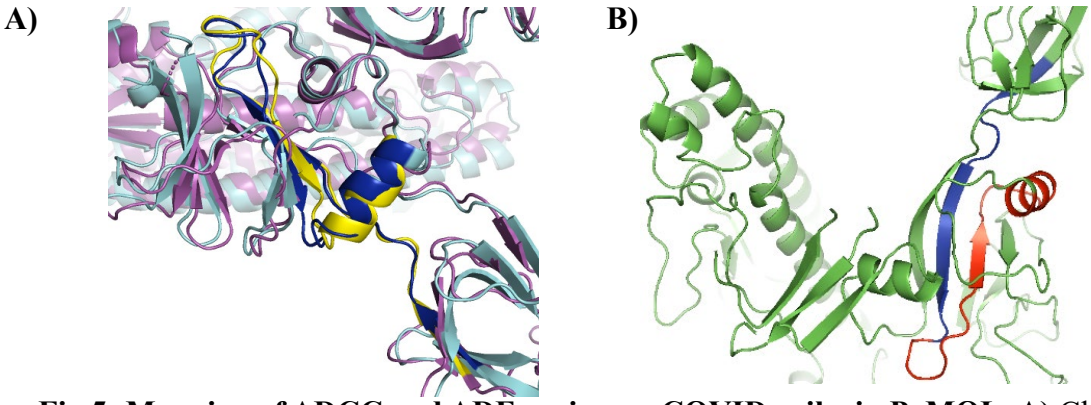


Fig 5: Mapping of ADCC and ADE region on COVID spike in PyMOL. A) Clustal O mapped region (585-625; blue) for COVID spike (cyan) is investigated by structural alignment on ADE inducing region (yellow) in SARS Spike (magenta). **B)** In vaccine construct designed, red segment (Δ600-624) was deleted as that will sufficiently weaken that region (rather a beta cage to encase any possible immunological proteins). Other non-ADE sequences are coloured as green.

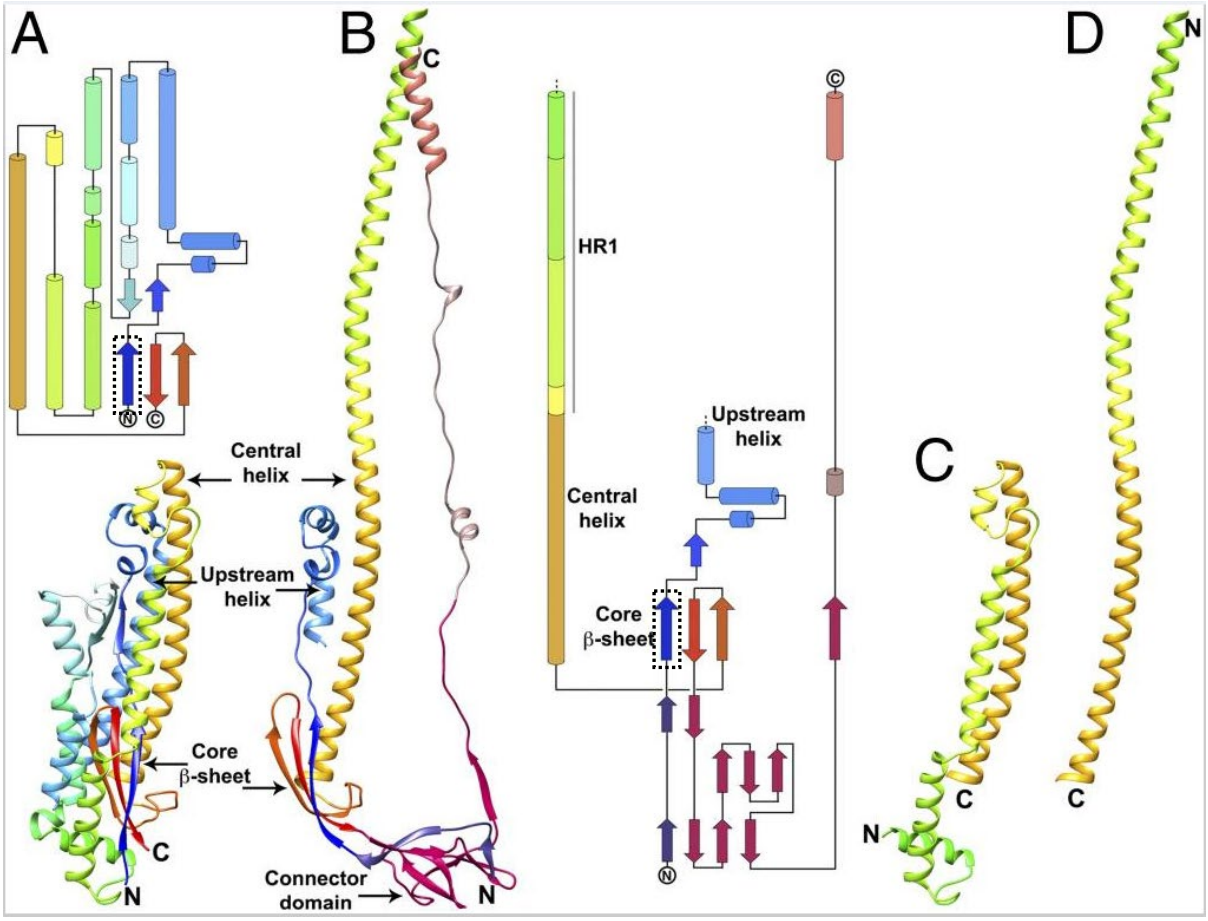


Fig 6: Core β-strand (dark blue, in dotted rectangle, residues: 711-729) is essential for pre- to post-fusion transition of spike. This strand maintains the core β-sheet by exchanging interactions with other two strands from same protomer to homologous strands of different protomer of trimeric spike. A) Pre-fusion spike S2 apparatus comprising central helix, HR region and core β-sheet. B) Post-fusion spike S2 apparatus comprising central

helix fused with HR region; Core β-sheet with exchanged 2nd and 3rd strands between protomers. Core β- strand remains unchanged. C) Central helix in pre and post-fusion conformation. [Reprinted with permission from PNAS (43)].

Final sequence

MFVFLVLLPLVSSQCVNLRTRTQLPPAYTNSFTRGVYYPDKVFRSSVLHSTQDLFLPFFS
NVTWFHVISGTNGTKRFDNPVLPFNDGVYFASIEKSNIIRGWIFGTTLDSKTQSLLIVNN
ATNVVIKVCEFQFCNDPFLDHKNNKSWMESGVYSSANNCTFEYVSQPFLMDLEGKQGNFK
NLREFVFKNIDGYFKIYSKHTPIIVEPERDLPQGFSVLEPLVDLPIGINITRFQTLLALH
RSYLTPGDSSSGLTAGAAAYYVGYLQPRTFLLKYNENGTITDAVDCALDPLSETKCTLKS
FTVEKGIYQTSNFRVQPTESIVRFPNITNLCPFDEVFNATRFASVYAWNRKRISNCVADY
SVLYNLAPFFTFKCYGVSPTKLNDLCFTNVYADSFVIRGDEVRQIAPGQTGNIADYNYKL
PDDFTGCVIAWNSNKLDSKVSGNYNYRYRLFRKSNLKPFERDISTEIYQAGNKPCNGVAG
FNCYFPLRSYSFRPTYGVGHQPYRVVVLSFELLHAPATVCGPKKSTNLVKNKCVNFNFNG
LKGTGVLTESNKKFLPFQQFGRDIADTTDAVRDPQTLEILDITPCSFGGVSVITHADQLT
PTWRVYSTGSNVFQTRAGCLIGAEYVNNSYECDIPIGAGICASYQTQTNSPRRARSVASQ
SIIAYTMSLGAENSVAYSNNSMTKTSVDCTMYICGDSTECSNLLLQYGSFCTQLKRALTG
IAVEQDKNTQEVFAQVKQIYKTPPIKYFGGFNFSQILPDPSKPSKRSFIEDLLFNKVTLA
DAGFIKQYGDCLGDIAARDLICAQKFKGLTVLPPLLTDEMIAQYTSALLAGTITSGWTFG
AGAALQIPFAMQMAYRFNGIGVTQNVLYENQKLIANQFNSAIGKIQDSLSSTASALGKLQ
NVVNHNAQALNTLVKQLSSKFGAISSVLNDIFSRLDKVEAEVQIDRLITGRLQSLQTYVT
QQLIRAAEIRASANLAATKMSECVLGQSKRVDFCGKGYHLMSFPQSAPHGVVFLHVTYVP
AQEKNFTTAPAICHDGKAHFPREGVFVSNGTHWFVTQRNFYEPQIITTDNTFVSGNCDVV
IGIVNNTVYDPLQPELDSFKEELDKYFKNHTSPDVDLGDISGINASVVNIQKEIDRLNEV
AKNLNESLIDLQELGKYEQYIKWPWYIWLGFIAGLIAIVMVTIMLEAAAKDLSPRWYFYY
LGTGPEAAAKNFGDQELIRQGTDYKHWPQIAQFAPSASAFFGMSREAAAKFYVYSRVKNL
NSSRVEAAAKQVILLNKHIDAYKTFPPTEPKKDKKKKADETEAAAKDEDDSE

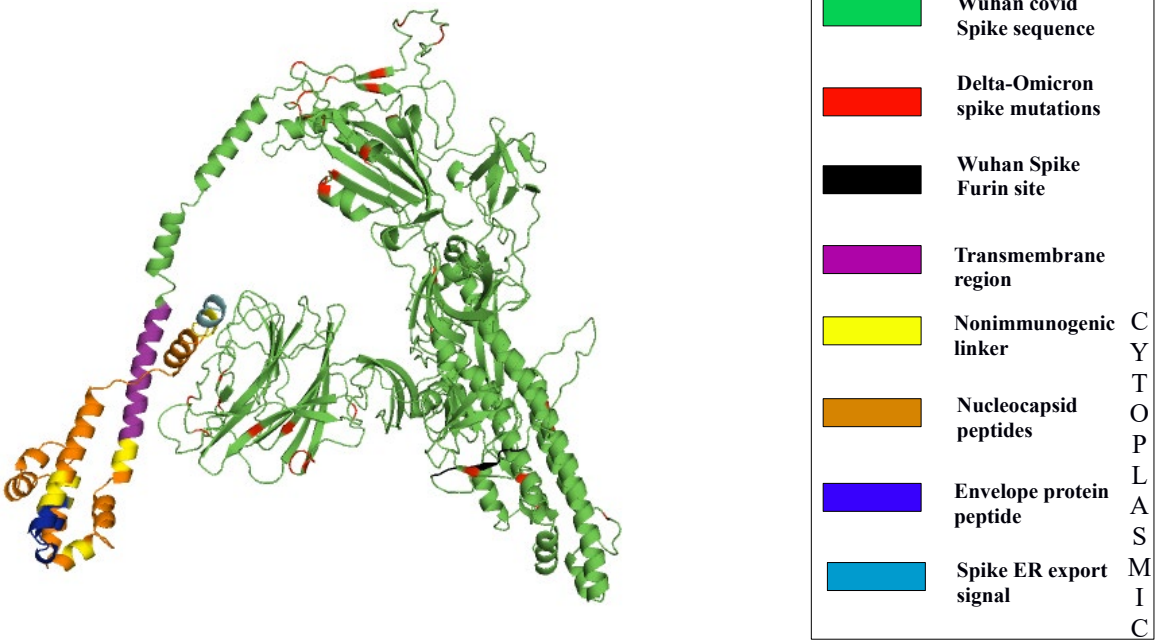


Fig 7: Sequence and 3-D model (RoseTTAFold) of the TM+ vaccine construct. The mutations corresponding to Delta and Omicron variants are coloured red. Transmembrane domain, linkers, cytoplasmic T-epitopes and terminal ER exit signals are coloured differently. All colour codes are explained in the legend on right side. The T-epitopes appear helical in this model. Overall the cytoplasmic domain looks like well folded coiled coil located close to the main protein.

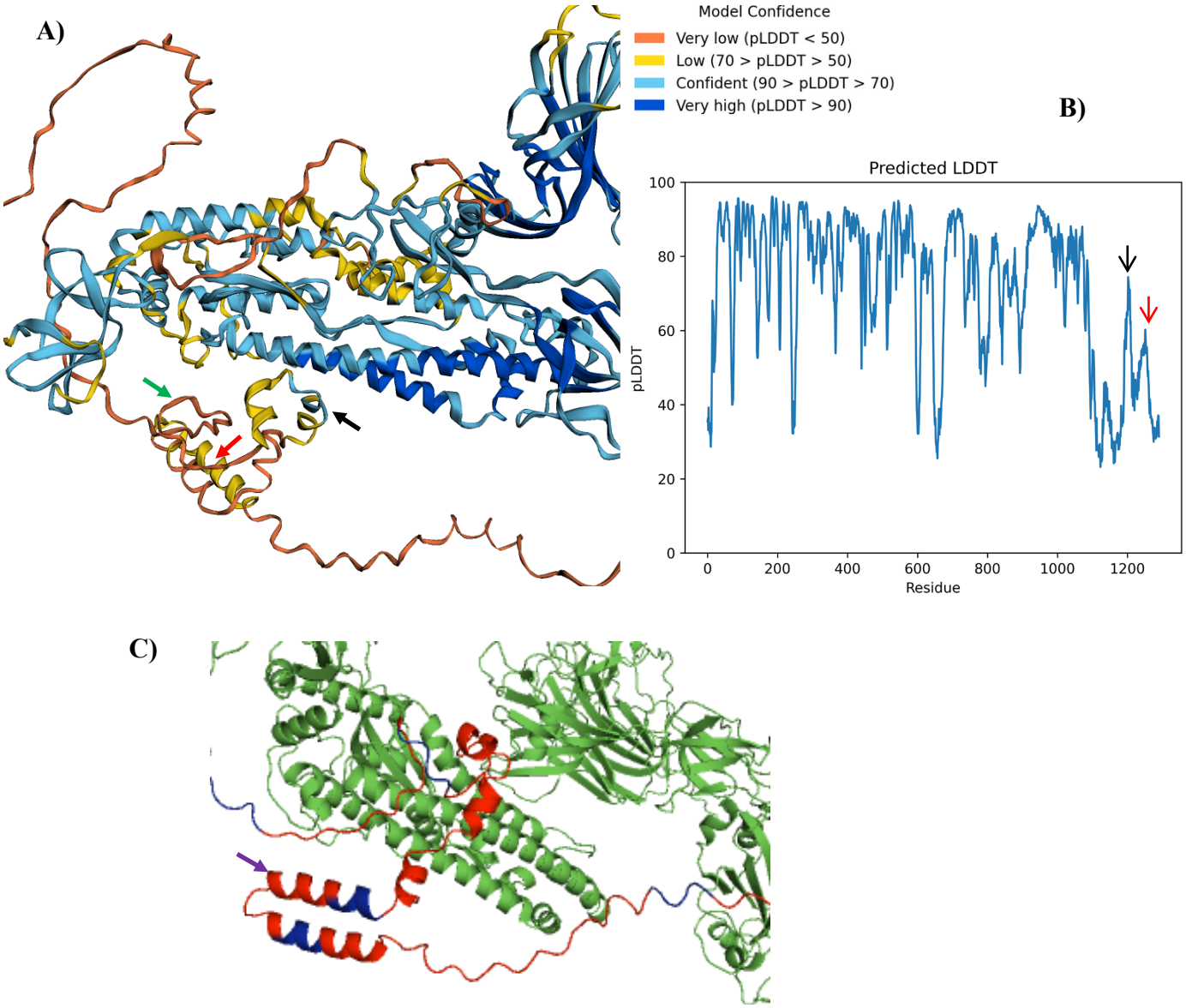


Fig 8: AlphaFold model of the final vaccine construct (without transmembrane domain) appears as well folded. A) Best model for the ΔTM construct and **B)** Predicted local distance difference test (pLDDT) plot. Except cytoplasmic domain, most of the other portions of the molecule have pLDDT values >70. The model is colour coded according to model confidence/pLDDT values in B. In cytoplasmic domain, the central region of folding driver epitope (marked by black arrow) has pLDDT value >70 and >50 for terminal portions. Similarly, part of memory peptide (red arrow marked) was folded with >50 pLDDT value. As expected from noncomplementary β-stranded structure in PDBsum, the first epitope (DLSPRWYFYYLGTGP) (green arrow marked) forms unstructured region (pLDDT<50). **C)** The envelope peptide between folding initiator peptide and memory epitope is folded as helices (from PyMOL; Purple arrow marked). The cytoplasmic epitopes and EAAAK linkers are coloured as red and blue respectively. Rest of the protein is coloured as green.

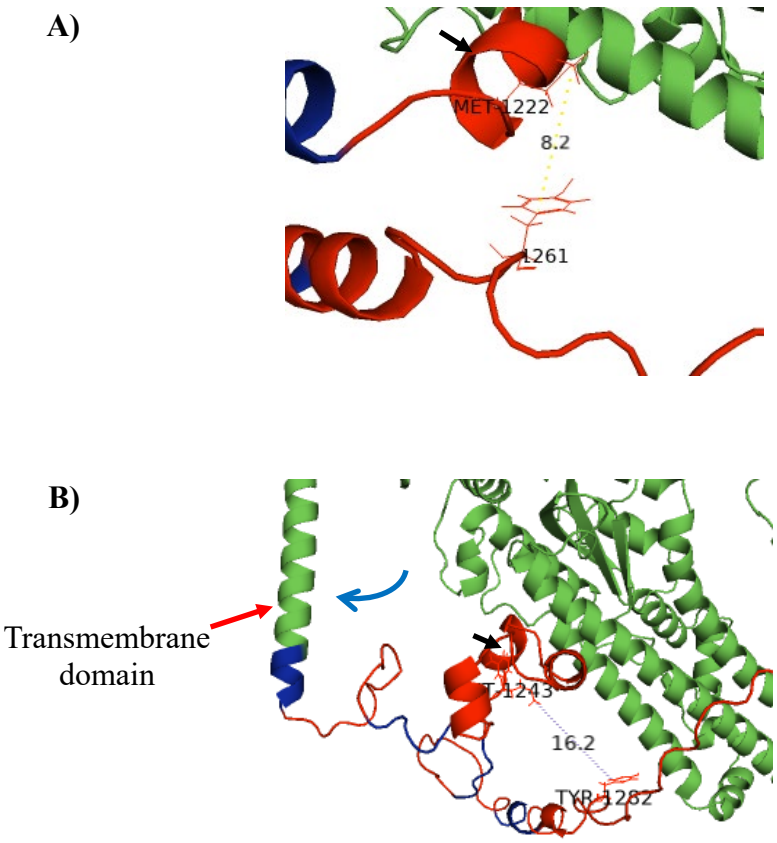


Fig 9: Folding centre of the vaccine constructs with T cell epitopes (red) joined by linkers (blue). **A)** Without transmembrane domain (ΔTM) and **B)** with transmembrane domain (+TM). The linkers (EAAAK) are coloured as blue while individual T-epitopes in cytoplasmic domain are coloured as red. The closest distance between folding driver epitope (marked by black arrow) and memory peptide is monitored by central methionine of the former and a tyrosine residue of the latter. In ΔTM, this distance is 8.2 Å which almost doubles to 16.2 Å in +TM construct. The transmembrane domain (marked by red arrow) is helical and seems to pull away (blue arrow) the cytoplasmic domain from rest of the protein which is coloured as green.

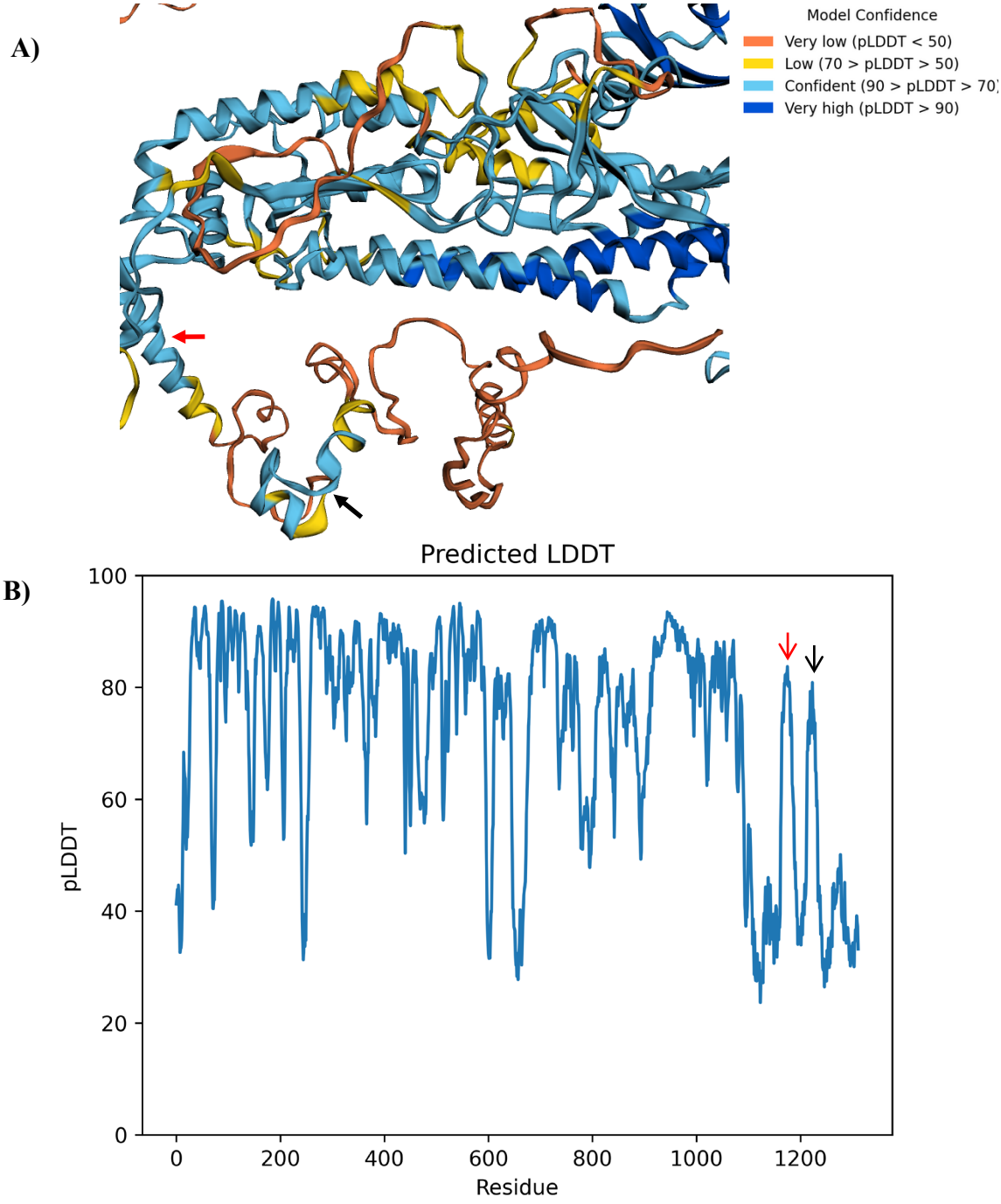


Fig 10: AlphaFold model of the vaccine construct with transmembrane domain (+TM) and predicted LDDT plot. A) The best model is shown. The folding initiator epitope is marked by black arrow. This epitope is well folded. Other epitopes are unstructured in cytoplasmic region. The transmembrane domain is marked by red arrow. **B)** The pLDDT plot shows that the red arrow marked transmembrane region (with pLDDT>80) and most of the other domains are well folded except cytoplasmic region. The model confidence for folding driver peptide (denoted by black arrow) is higher (≈80) than ΔTM indicating no effect from neighbouring unstructured epitopes.

Author Contributions:

AG and MMG designed the study. AG performed the AlphaFold data analysis and other Bioinformatics studies. MKS processed sequences by scripting to get the AlphaFold data from UPPMAX, Uppsala University, Sweden. AG and MMG wrote the paper.

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