



Immuno-informatics approach to design a multi-epitope vaccine to combat cytomegalovirus infection

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ABSTRACT

Human cytomegalovirus (HCMV) poses a serious public health problem causing morbidity and mortality in transplant recipients, immunocompromised patients, and congenitally infected newborns. Considering the recent reports of emergence of Ganciclovir drug resistance, vaccine development is the need of an hour. In the present study, a multi-epitope vaccine was constructed targeting the major hotspot- the pentavalent complex of glycoproteins (H/L/UL128-UL130-UL131) of HCMV, and other important target proteins- gB and pp65. The vaccine designed was composed of series of epitopes belonging to CD4, CD8 and B cells. As an immunobooster, the CpG motifs was linked to the vaccine which served as an adjuvant. The affinity, stability and flexibility of the vaccine construct with the immune receptor- Toll like receptor -9 (TLR-9) was investigated by molecular docking and molecular dynamics simulations. The *in-silico* immune simulations of the vaccine sequence were also carried out to determine its ability to stimulate different immune components. Further, an *in-silico* cloning of the vaccine construct was performed to analyze the feasibility of its expression and translation efficiency in pET-28a (+) vector. The overall results obtained indicated the vaccine to be immunogenic, non-allergic, had high population coverage, high affinity and stability with the immune receptor, had efficient expression in host *E. coli* and was effective in stimulating different immune cell types like T helper, T cytotoxic, B cells, dendritic cells, macrophages and interleukins. The proposed vaccine construct is expected to be highly efficacious and needs to be carried forward by the vaccinologists to test its efficacy in lab settings.

1. Introduction

Cytomegalovirus is a ubiquitous human herpes virus belonging to family *Herpesviridae* with a seropositivity of >60% in adults worldwide (Ciferri et al., 2015). The infection caused by HCMV is usually asymptomatic, however, can cause severe complications or even death in patients with hematopoietic stem cell transplant, solid organ transplant and patients who are immunocompromised. In addition, HCMV also causes severe birth defects in the developing fetus, as it can infect the placenta by crossing the barrier (Britt, 2008). Moreover, the risk of organ graft failure is increased in HCMV (Landolfo et al., 2003). HCMV can also infect wide range of tissues and organs like dendritic cells, hepatocytes, neurons, macrophages, leukocytes, epithelial cells, endothelial cells and fibroblasts (Singer et al., 2008). Keeping into account the severity of diseases and the ability to infect wide range of organs, an effective vaccine against HCMV is considered a public health priority (Modlin et al., 2004). Considering the recent reports of emergence of drug resistance to ganciclovir in HCMV (Lurain, and Chou, 2010; Fisher et al., 2017), an effective vaccine is the need of an hour.

Just like other herpesviruses, the conserved fusion machinery formed by the envelope glycoproteins gB and gH/gL are required for viral entry (Heldwein et al., 2006; Connolly et al., 2011). In addition, the glycoproteins-gH/gL/UL128/UL130/UL131A forms the pentameric complex which is required by HCMV for entry into both epithelial and endothelial cells (Wang and Shenk, 2005; Ryckman et al., 2006; Ryckman et al., 2008) and the glycoprotein B (gB) facilitates in viral entry by mediating the fusion of virus with host membrane (Heldwein et al., 2006) (Fig. 1). An effective neutralizing humoral response in hosts against this pentamer has been demonstrated by several research groups, thereby indicating the importance of these proteins in vaccine designing. Several studies have demonstrated strong neutralizing humoral response by this pentameric complex in epithelial/endothelial cells (Genini et al., 2011; Fouts et al., 2012; Freed, 2013). The strong neutralizing antibodies response against this pentamer has been demonstrated by several research groups thereby indicating it to be an important target for vaccine development (Wussow and Diamond, 2013; Fu et al., 2012; Wen et al., 2014). Thus, we aimed to identify the immunogenic regions in this glycoproteins pentavalent

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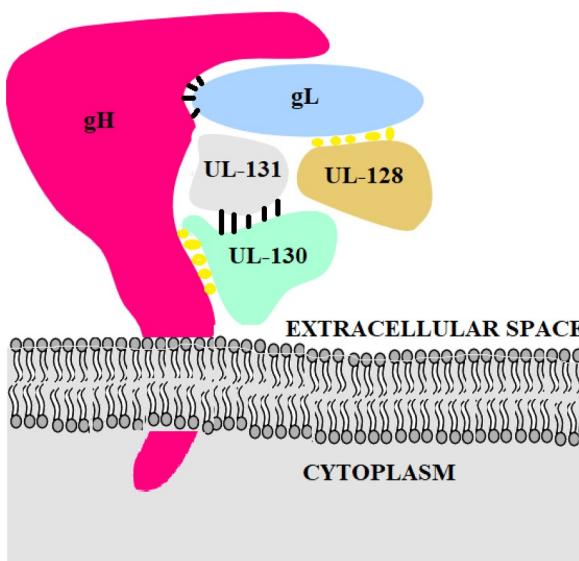


Fig. 1. Model showing the pentamer complex formed by the glycoproteins-gH/gL/UL128/UL130/UL131 which promotes the entry of viruses into the epithelial cells .

complex of HCMV which facilitates its entry and gB and the tegument protein- pp65. Due to an accumulation of large amount scientific of data in the form of literature, genomic sequences and databases, it is possible to identify the potential immunogenic markers in the protein sequence, in the form of T and B cell epitopes, and to leave behind the remaining protein sequence which may un-necessarily increase the vaccine load. This technology has led to an emergence of new field of science known as immunoinformatics, which is a combination of experimental immunology and computer science (Tomar and Dey, 2018). The recently proposed recombinant vaccine for malaria -RTS,S/AS01 by GlaxoSmithKline, contains T cell epitopes (Pance, 2019). Such peptide based immunotherapies using the immunoinformatics approaches have been proposed by several research groups against numerous infectious diseases like Hepatitis C virus, Herpes Simplex virus, Zika virus, Dengue virus, Human Immunodeficiency virus etc. (Chauhan et al., 2018a, 2018b; Alam et al., 2016; Ali et al., 2017; Pandey et al., 2018). In the present study, a multiepitope vaccine was designed containing the epitopes which could induce the activation of several immune cells including -T (Tc & Th) cells, B cells and IFN- γ cells, thus could provide an effective immunity to host.

2. Materials and methods

2.1. Target proteins, analysis of various physicochemical and structural properties

The amino acid sequences of gB, gH, gL, UL-123, UL-128, UL-130, UL-131 and pp65 of HCMV were obtained from NCBI database (<https://www.ncbi.nlm.nih.gov/protein>). The Expasy Protparam tool (<http://web.expasy.org/protparam/>) was used for analyzing the physicochemical properties of the proteins. Further, in order to determine the antigenicity of the proteins, the VaxiJen 2.0 server was utilized, with the threshold set at 0.4 (Doytchinova and Flower, 2007). The server was designed to overcome the several limitations of the alignment based antigenicity prediction methods and uses the auto cross covariance (ACC) based method for computation. The secondary structural properties were determined using SOPMA online tool without altering any parameter (Geourjon and Deleage, 1995).

Since the three dimensional (3D) structures of the target proteins were not present in the protein data bank (PDB), thus were modelled using well-known and widely used homology modeling tool- Raptor X

(Kallberg et al., al.,2012). Raptor X server is CASP10 template based modeling system with improved prediction of target protein structures which have even less than 30% sequence identity with the structures available in PDB. It was observed that the server outperformed all the servers based on CASP10 template based modeling system including consensus and refinement based methods. Further the refinement of the 3D models was carried out using ModRefiner tool (Xu, and Zhang, 2011). Next, the finalized 3D models were subjected to MolProbity server, to determine their quality and reliability using Ramachandran plot analysis (Chen et al., 2010).

2.2. T cell epitopes prediction

2.2.1. Helper T-lymphocyte (HTL) epitopes

The 15-mer epitopes HTL (CD4) epitopes were identified using IEDB consensus methods and Net MHC II pan 3.2 server. The HLA Class II alleles included for prediction of epitopes were: DRB1*- 01:01, 03:01, 04:01, 04:02, 04:03, 04:04, 04:05, 07:01, 07:03, 08:01, 08:03, 09:01, 09:02, 09:03, 10:01, 10:02, 10:03, 11:01, 12:01, 13:01, 13:02, 14:01, 14:02, 15:01, 15:02, 15:03, DRB3 × 02:02, DRB5 × 01:01, DQA1 × 01:02-DQB1 × 06:02, DPA1 × 02:01-DPB1 × 01:01 and DPA1 × 02:01-DPB11 × 14:01 (Jensen et al., 2018; Wang et al., 2010). Net MHC II pan 3.2 is an updated version for prediction of epitopes over previously used NetMHCII methods, with extended data sets used for prediction of quantitative analysis of HLA-epitope binding affinity. Similarly, the IEDB consensus methods outperformed the recently developed NN-align based epitope prediction method. Thus, the servers employed for prediction of epitopes in the study were recently updated and improved versions with better prediction capabilities. The alleles included are prevalent worldwide with an expected coverage of more than 95% population (Moise et al., 2009). The epitopes were further categorized as strong binders with the percentile rank of 2%, and intermediate and non-bindlers with 10% and >10% ranks respectively.

2.2.2. Cytotoxic T-lymphocyte (CTL) epitopes

The 9-mer CTL epitopes were predicted using the NetCTL 1.2 server, targeting the commonly occurring HLA Class I supertypes (Larsen et al., 2007). The server is an updated version to 1.0 with improved prediction of HLA-epitope binding and improved proteasomal cleavage prediction. In addition, the IEDB consensus method was used for predicting the epitopes against some additional HLA Class I alleles, which are prevalent in human population i.e. A-02:01 and B-35:01, 51:01 & 58:01 (Kim et al., 2012). More than 90% of the worldwide population were expected to be covered by the alleles included in the study (Moise et al., 2009). Only the epitopes predicted as strong binders (with consensus score of ≤ 2) were considered for further analysis.

2.2.3. Analyzing the promiscuity and overlapping nature of the screened out epitopes

The promiscuous epitopes are able to bind and induce the activation of multiple allelic forms of HLAs, therefore, an effective immune response could be generated in host by utilizing such epitopes. Thus the promiscuity analysis of the screened out HLA T cell epitopes was carried out.

The overlapping epitopes are composed of inbuilt sequences targeting both HLA Class I (CTL) and HLA Class II (HTL) cells, thus have the potential to activate both T cell subtypes. Thus, we listed out the HTL and CTL epitopes of high binding affinity which overlapped and merged them as a single peptide fragment.

As such epitopes (promiscuous and overlapping) will have the ability to interact with maximum number of HLA alleles, the possibility of high population coverage by the screened out epitopes will thus be increased.

2.3. Prediction B cell epitopes (linear and conformational)

The B cell lymphocytes differentiates into memory and plasma cells on encountering with the B cell epitopes, thus are important from vaccine perspective. Thus, the B cell epitopes (linear/continuous) were predicted using BCpred 2.0 and BepiPred 2.0 servers prediction (El-Manzalawy et al., 2008; Jespersen et al., 2017). Only the epitopes with the threshold of 0.8 and above were carried forward for further analysis. In addition, the Ellipro server (threshold set at 0.7) was used for predicting discontinuous/conformational epitopes (Ponomarenko et al., 2008).

2.4. Characterization of screened out epitopes

The conservancy and the population coverage of the predicted promiscuous epitopes were analyzed by IEDB Conservancy Analysis tool and IEDB population coverage analysis (Bui et al., 2007, 2006). The physicochemical properties of the screened out conserved epitopes was examined using Expasy ProtParam tool. Further, the epitopes were also screened for having any similarity against human proteome using the BLASP server.

2.5. Modelling the immune receptor

Since CMV is a ds DNA virus, the dendritic cells and B cells with TLR-9 expressed on their surface will primarily recognize its structural components. Thus it is important that the vaccine designed should have affinity for TLR-9 and should aid in its activation. Thus the UniProt (ID-Q9NR96) was used for retrieval of TLR-9 amino acid sequence which was further subjected to an online homology modeling tool- Raptor-X. The server predicts the target proteins 3D model, disordered and solvent accessibility regions. The models generated are indicated by scores like GDT and uGDT which predicts the model's absolute global quality, P-value measures the absolute local quality and RMSD measures the relative global quality respectively. The model was further subjected for refinement, quality and reliability testing as described above.

2.6. Designing the multi-epitope vaccine

2.6.1. Sequence construct

The sequence construct of the vaccine was composed of B cell, HLA Class I (CTL) and HLA Class II (HTL) epitopes. Before inclusion of the epitopes in the multi-epitope vaccine construct, they were passed through several immune filters. The filters designed were, a) the epitopes should be promiscuous; (b) should have overlapping sequences; (c) immunogenic; (d) should be 100% conserved (e) non-allergic; and (f) should not have any similarity with the human proteins. Considering the following criteria, the HTL, B cell and CTL epitopes were screened out, and were merged using suitable linkers to form a single peptide fragment. An adjuvant (CpG-containing oligodeoxynucleotides) was also attached via EAAAK linker for boosting the immune response of the vaccine.

2.6.2. Assessment of physicochemical properties

The vaccine's amino acid sequence was analyzed for its antigenicity and allergenicity using VaxiJen v2.0 and AlgPred servers. Further, the ProtParam online tool was used for analyzing other physicochemical parameters like grand average of hydropathicity (GRAVY), instability and aliphatic index, molecular weight, half-life, theoretical isoelectric point (pI) and amino acid composition. IFN- γ is an important marker for vaccine perspective as it is well known in eliciting an effective immune response against tumor and viral infections. Thus, the vaccine construct was also scanned for IFN- γ inducing epitopes to analyze whether it can induce the IFN- γ cells activation or not using IFNepitope server (Dhanda et al., 2013).

2.6.3. Modeling, refinement and validation of the vaccine

The secondary structural properties and 3D structure of the vaccine construct was analyzed and modelled using SOPMA server and the Raptor-X homology modeling tool respectively. Further, the model was subjected to refinement for improving its structural quality utilizing Galaxy Refine server. Among the algorithms known for refinement and improving the structural quality of any constructed 3D model, it is one of the top performing tool (Heo et al., 2013). This server relaxes the modeled structure on the basis of CASP10 associated refinement technique. Further the validation of the refined vaccine construct tertiary structure was done by ProSA-web server (Wiederstein, and Sippl, 2007). The overall quality of the model predicted by the ProSA-web server should lie within the range of z scores, characteristic for native proteins. The quality of the model is considered erroneous if the score doesn't lie within the range. Further, the refined was subjected for Ramachandran plot analysis model in order to assess its quality using MolProbity server. Further the flexibility and plausible direction of the motion of the vaccine model was analyzed using the recently updated CABS-flex 2.0 server. The server provides the simulated molecular motion/fluctuation of the protein w.r.t its flexibility and stability (Kuriata et al., 2018).

2.7. In-silico Immune simulation by the vaccine construct

In order to analyze that whether the vaccine construct is capable of generating an effective immune response against HCMV, an *in-silico* immune simulation was carried out using Position Specific Scoring Matrix (PSSM) based C-ImmSim 10.1 server (Rapin et al., 2010). The server simulates the three different anatomical regions i.e. bone marrow, thymus and lymph node, the three major compartments found in mammals. The potency of the vaccine sequence to simulate different immune cell types like T-helper, T-cytotoxic, B cells, NK cells, macrophages, dendritic cells, Immunoglobulins and cytokines was investigated. The time set for injections were not altered and were set as fault i.e. 1, 84 and 168, the time set for 1st injection was 0 and the each time set was 8 h. Therefore, 3 injections were provided at 4 weeks interval. The simulation was run at 1000 simulation steps at a random seed.

2.8. Molecular docking and molecular dynamics simulations between the multi-epitope vaccine and the immune receptor

An effective immune response is generated in host if there is a proper interaction between an antigen/vaccine and the target immune cell. Thus, the interaction patterns between vaccine construct (ligand) and the immune receptor (TLR-9) was analyzed using ClusPro, an online web molecular docking tool. The results obtained after molecular docking analysis were visualized in Pymol (Schrodinger). In addition, the HLA T cell epitopes (both HTLs and CTLs) interaction patterns with the commonly occurring HLA Class II- DRB1 alleles in human population i.e. *01:01 and *15:01 (with PDB IDs- 2 g9h and 1bx2) and HLA Class I allele - A*02:01 (PDB ID- 1qew) were also analyzed respectively.

Further, the molecular dynamics simulation was carried out using Desmond tool (Schrodinger) for determining the stability and flexibility in the interaction patterns of the docked complex (b/w TLR-9 and vaccine) at the microscopic level. The docked complex was enclosed in a buffer filled cubic box at a distance of 10 Å and the simulation parameters were set close to the human physiological conditions, like the physiological pH, neutralization of the system by adding Na⁺ ions, salt concentration (NaCl) of 0.15 M etc.; and were looked for the RMSF and RMSD plots at the time duration of 20 nanoseconds.

In addition, the iMODs server was utilized for analysing the stability of vaccine construct and immune receptor docked complex (López-Blanco et al., 2014). It determines the collective motions by normal mode analysis (NMA) in internal coordinates of the complex. The results are represented in terms of B-factor/Mobility, Variance,

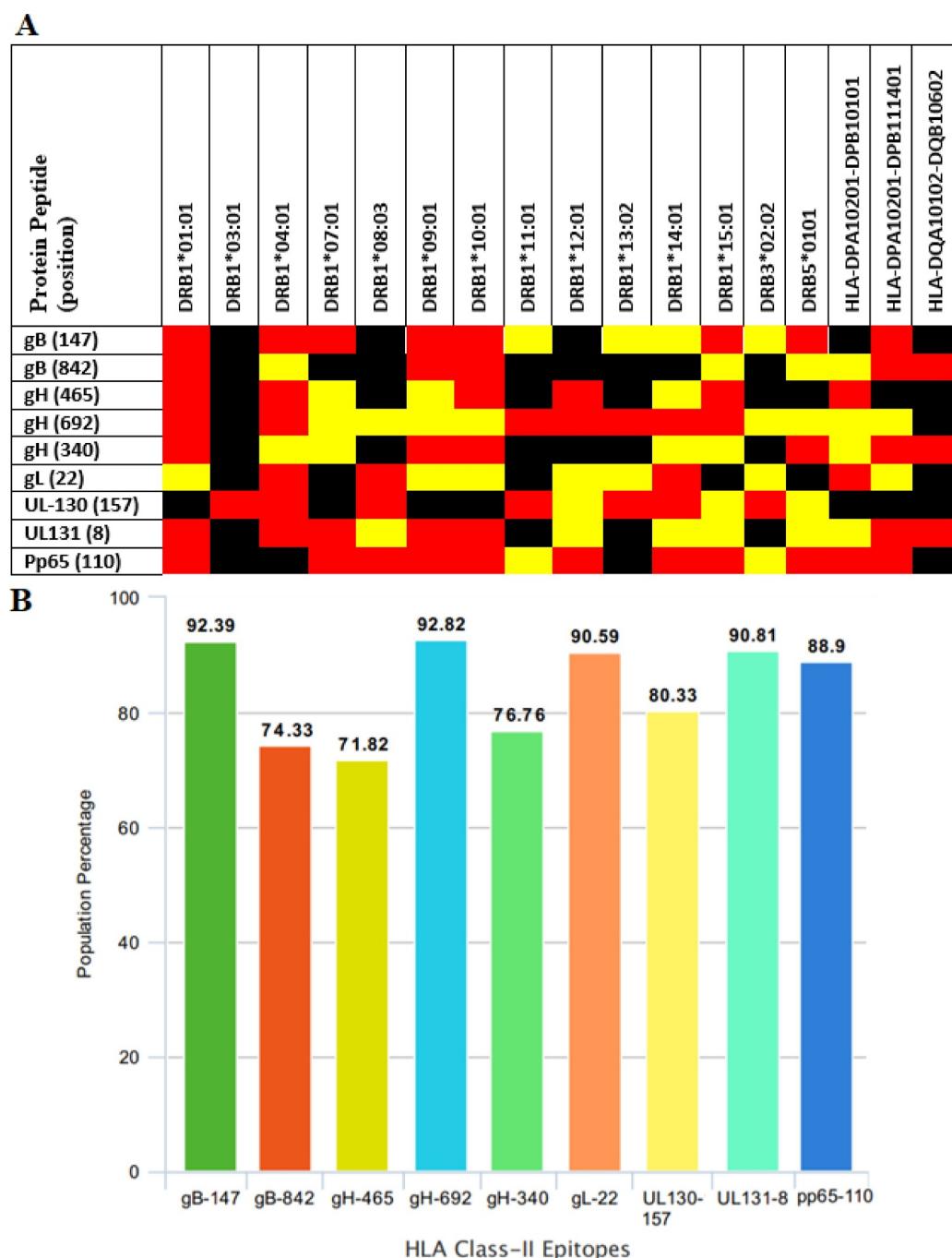


Fig. 2. (A)- Heat map showing the high promiscuity of the screened out HLA Class II (HTL) T cell epitopes. Red color boxes indicates the high binding affinity, yellow- low binding and black non-binding epitopes respectively. However, only some of the important HLA alleles are shown in the figure. For more details kindly refer Table S4. (B)- The population coverage of the respective screened out HLA Class II epitopes. .

Eigenvalues, Elastic network and Covariance map.

2.9. Cloning and optimization of the vaccine sequence

The cloning and optimization of the amino acid sequence of the vaccine was carried out using an *in-silico* tool- Java Codon Adaptation Tool (JCat) (Grote et al., 2005). The tool was used for performing the reverse transcription of the of the vaccine sequence, its codon optimization and expression in *E. coli* - K12 strain, expression vector. The ideal value of codon adaptation index (CAI) as calculated by the JCat tool is 1.0. It also measures the vaccine insert's GC content, which should range ideally between 30–70% or else, the efficiency of transcription

and translation may be unfavorable. The optimized sequence of the vaccine was finally inserted into the expression vector (pET-28a (+)) utilizing SnapGene tool.

3. Results and discussion

3.1. Analysis of structural and physicochemical properties of the vaccine candidate proteins

The accession numbers of the amino acid sequences retrieved from NCBI database are shown in Supplementary Table 1. The Blastp (protein-protein BLAST) database: Reference proteins (refseq_protein) was

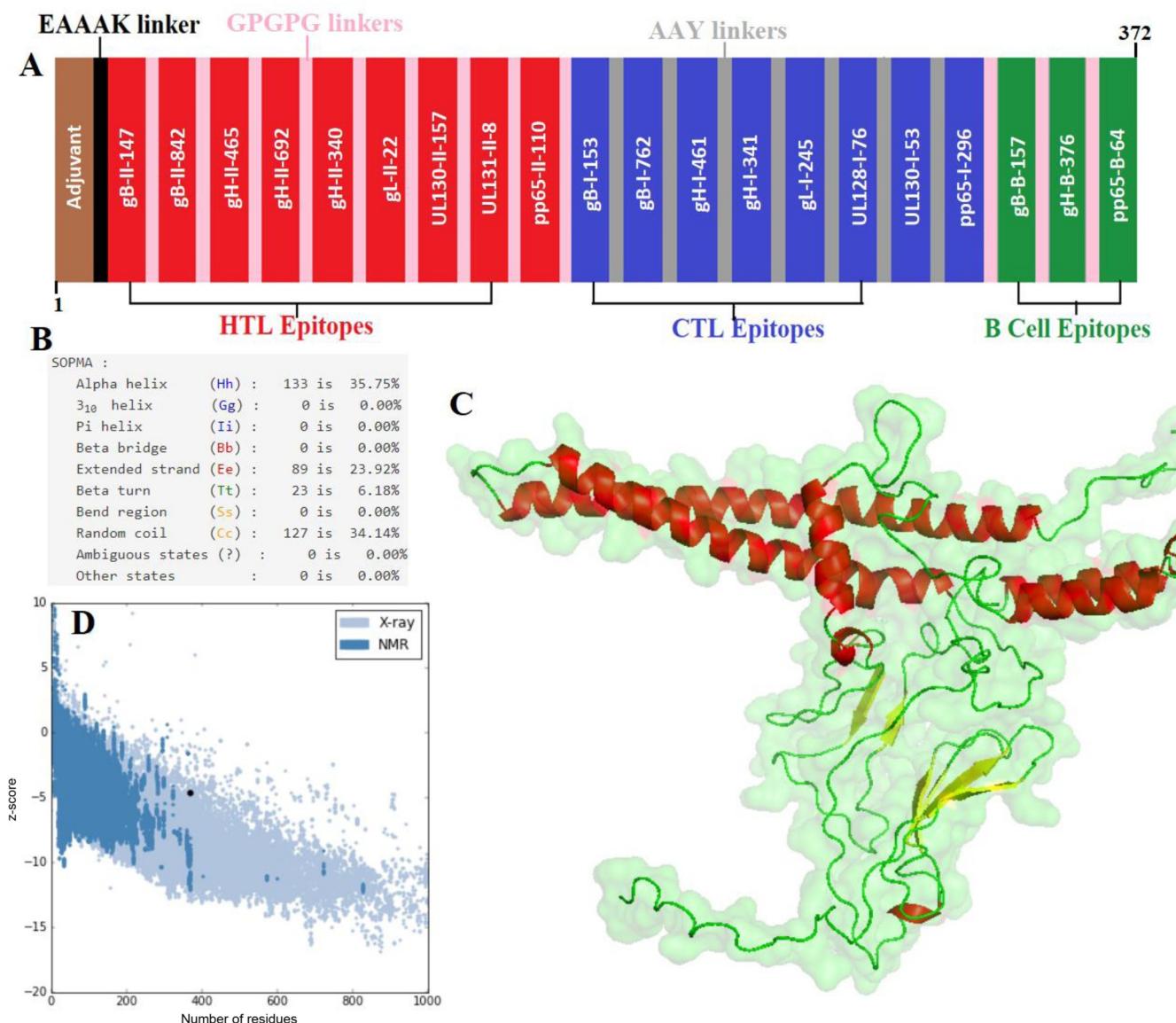


Fig. 3. (A)- Schematic representation of multi-epitope vaccine construct. The HLA Class II, Class I and B cell epitopes are indicated in red, blue and green color respectively. The GPGPG linkers (light pink color) were utilized for linking the HLA Class II and B cell epitopes and AAY linkers (gray color) for linking the HLA Class I epitopes. Likewise, EAAAK linker (black color) were utilized for linking the adjuvant to the epitope chain (B)- Secondary structural properties of the vaccine construct. (C)- Tertiary structure of vaccine. The helical, sheet and loop regions are represented in red, yellow and green colors respectively. (D)- PROSA results, validating the 3D modelled vaccine structure. .

used for identification of any hits between the target proteins of HHV-5 with the human proteome without altering any parameters. None showed any similarity with human proteome. Among the target proteins, the antigenicity score of pp65 was found to be 0.57, thus was predicted to be most antigenic, followed by UL-131 (0.56), gB (0.52), UL-128 (0.47), gH (0.46), UL- 130 (0.43), gL (0.36) and UL-123 (0.33). This indicates that in future if someone wants to design the whole protein based vaccine, then one might think of pp65, since it was predicted to be most antigenic and other proteins in the subsequent order based on the antigenicity scores. The secondary structural properties and other physicochemical properties of each target protein was also analyzed (Supplementary Figure 1, Supplementary Table 2). The 3D models of each protein was modeled using an online homology modeling tool Raptor X. Further the retrieved models were refined using Modrefiner server. The reliability and quality of the finalized models were checked using Ramachandran plot analysis (Supplementary Table 3). All the models generated showed higher number of amino acids in allowed region, thus indicating their good

quality. These models were prepared for two reasons. Firstly, to map the epitopes on the respective proteins 3D models, to identify and confirm their surface location. Secondly, for prediction of conformation B cell epitopes using Ellipro server, as the server predicts the conformational epitopes using the 3D model of the protein.

3.2. CD4, CD8 and b cell epitopes prediction

The CD4 and CD8 epitopes were identified using the online web tools- Net MHC II pan 3.2, IEDB consensus and NetCTL1.2 servers respectively. The criteria designed for predicting the best possible T cell epitopes (both CD4 and CD8) was that they should not have any similarity with the human proteins, should be promiscuous, should be 100% conserved, and should not lie within the post translational modification sites and glycosylation sites of the respective proteins,. Based on all these parameters, some promiscuous and overlapping epitopes were identified in the present study (Supplementary Table 4, 5 & 6).

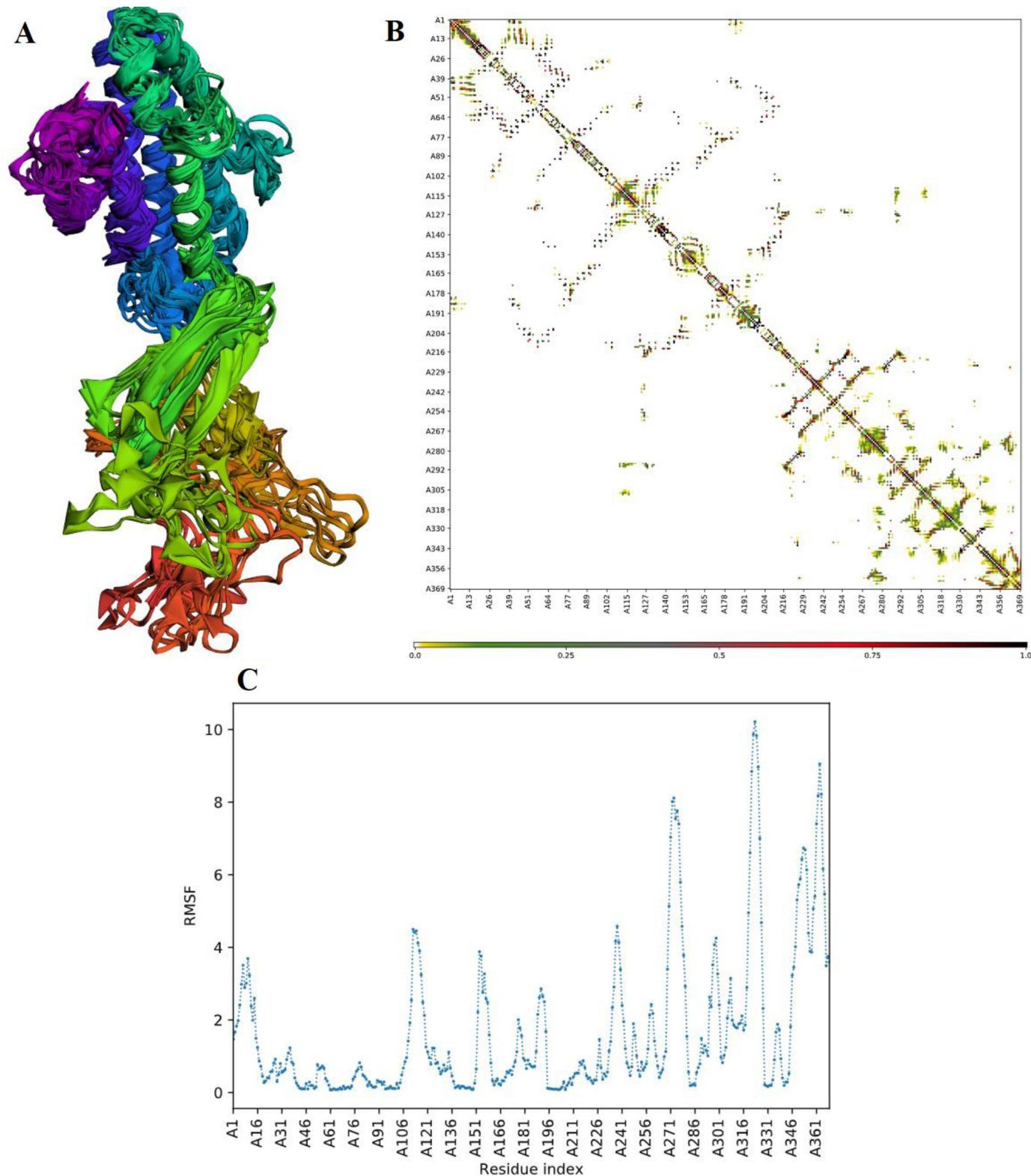


Fig. 4. CABS-flex 2.0 results. A- The ten final models (represented as cartoons) generated showing minor fluctuations. The major fluctuations were observed in the red and brown regions. B- Contact map, representing the detailed view of the interaction between the residue-residue of protein. The interactive area is represented in the central panel. Note the latter half of the sequence shows better interactive patterns where the epitopes are located. C- Fluctuation plot representing the fluctuations in the residues during simulation.

The BCpred 2.0 and BepiPred 2.0 online tools were employed for predicting linear/continuous B cell epitopes. The maximum number of B cell epitopes were predicted in gB protein i.e. 18, followed by pp65-11, gH & UL-123- 9, UL-130- 5, gL-4, UL-128- 3 and UL-131- 2. The

conformational/ discontinuous B cell epitopes were predicted by Ellipro server. gH was predicted to have 5 conformational B cell epitopes followed by gB (3), gL (2), UL123 (2), UL128 (2), UL130 (2), pp65 (2) and UL131 (1) (Supplementary Figure 2).

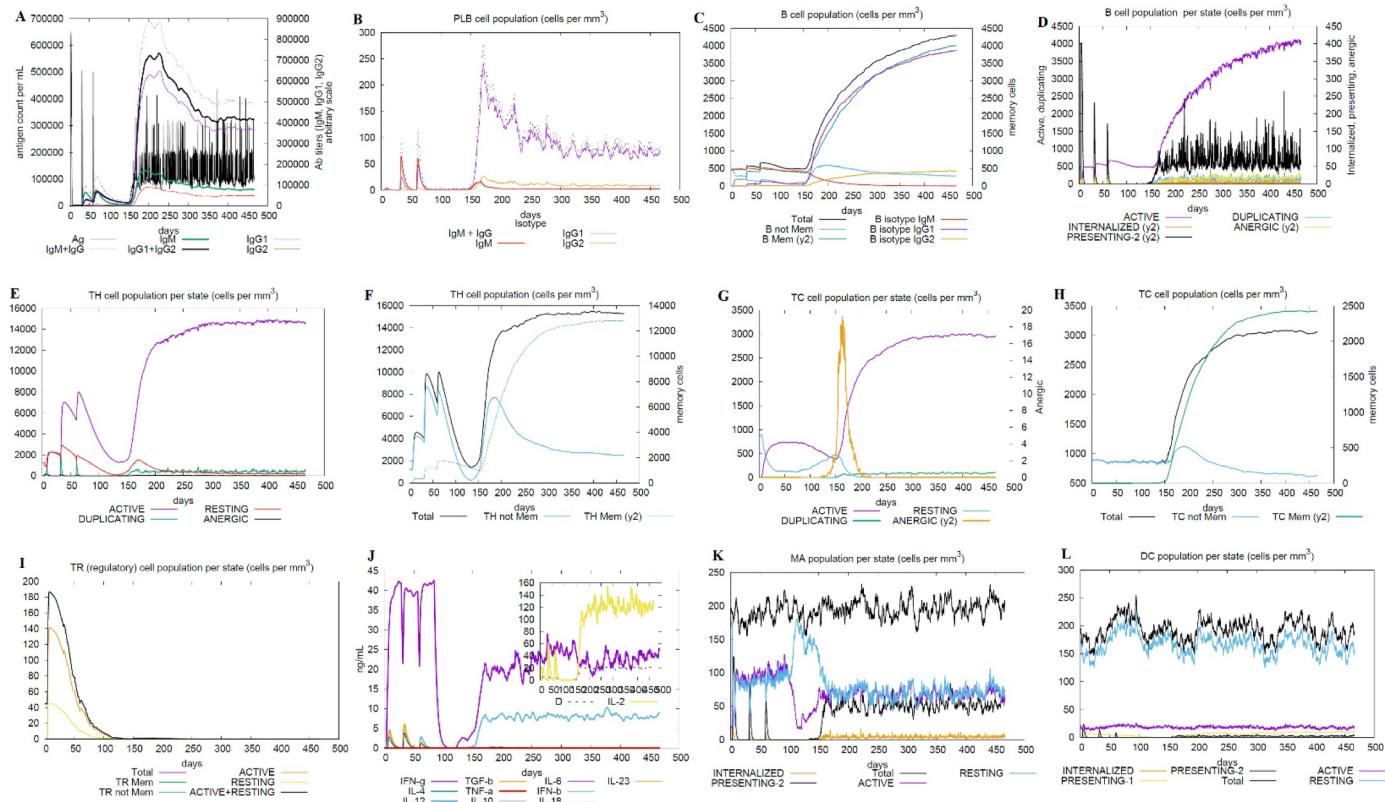


Fig. 5. The in-silico immune simulation results by the candidate vaccine. A to D- Immunoglobulins and B cell production and formation of memory B cells; E&F- T helper cells production; G & H- T cytotoxic cells production; I- Reduced levels of T regulatory cells; J- Different cytokine levels; K- Levels of macrophages; and L- levels of Dendritic cells .

3.3. Multi-epitope vaccine construction

Before inclusion of the epitopes in multi-epitope vaccine sequence, they were passed through several immune filters. Only the epitopes which fulfilled the designed filtering parameters were selected. The epitope filtering parameters designed were : (a) they should have overlapping HLA Class II (HTL) and HLA Class I (CTL) epitopes, (b) should be promiscuous (Fig. 2A) (c) high population coverage (Fig. 2B) (d) high binding affinity with HLAs (by docking analysis) and (e) should not elicit autoimmunity i.e. should not share sequence similarity with any of the human genes. Based on these parameters, 9 HTL, 8 CTL and 3 B cell epitopes were screened out for inclusion in the vaccine construct. The surface localization of the epitopes was also checked by mapping them on their respective 3D models (Supplementary Figure 3). The HTL/CD4 epitopes were linked by GPGPG linkers and CTL/CD8 epitopes were linked by AAY linkers. For boosting the immune response of the vaccine, the CpG-ODNs (adjuvant) were also attached via EAAAK linker to the following epitope chain (Fig. 3A). The promising adjuvant capabilities of the CpG-containing oligodeoxynucleotides have been reported previously by several research groups against number of infectious diseases like hepatitis B virus, measles virus, orthopox viruses cancers, influenza virus, lymphocytic choriomeningitis virus, etc. (Krieg, 2011). These ODNs are of mainly four types: the most widely studied and tested K-type, C-type, D-type, and P-type. In humans in clinical trials, the adjuvant capabilities of K type ODNs has been tested against cancer and number of infectious diseases (Steinhagen et al., 2011). Thus, in our final multi-epitope construct, we implemented the K-type CpG-Oligonucleotides (ODNs), which served as an adjuvant. The vaccine constructed was composed of 372 aa's. The secondary structural properties as retrieved by SOPMA server of the vaccine construct is shown in Fig. 3B. The vaccine was modeled via multi-template mode. The template used for construction of a multiepitope vaccine model

were 3v0cA, 1epwA and 3zurA (Fig. 3C). Further, the 3D model of vaccine was refined using Galaxy Refine server, to improve its overall quality. Finally, the quality of the finalized vaccine model was checked by ProSA-web and Ramachandran plot analysis. The z score observed for the vaccine model was -4.64 by ProSA-web, which was found within the score range of native proteins with comparable size (Fig. 3D). The Ramachandran plot results revealed 92.7% residues in favored region and 98.9% residues in allowed regions (Supplementary Figure 4).

The molecular motion analysis of the vaccine provided by the CABSflex 2.0 server revealed the flexible and rigid portions of the vaccine construct. Among the 10 final 3D structures retrieved, the starting positions showed lesser fluctuations where an adjuvant was linked whereas the fluctuations were observed towards the other end where a series of epitopes were attached via linkers (shown in dark brown and red regions) (Fig. 4A). The contact map showed the residue-residue interaction pattern for all the 10 models (Fig. 4B). The root mean square fluctuation plot revealed the fluctuation of each of the residue of vaccine ranging from 0 Å to 10 Å. The fluctuations were higher towards the latter half of the sequence which was mainly comprised of series of epitope, thus indicating the high flexibility of the vaccine (Fig. 4C).

The *in-silico* immune system simulation by the vaccine sequence showed higher activity of T and B cells, which was in accordance with the actual immune responses. The increased levels of IgM in the beginning was indicative of the primary immune response followed by the higher levels of IgM, IgG, IgM + IgG and IgG₁ + IgG₂ in secondary and tertiary reactions with decrease Antigen concentrations. The higher levels of activated B cells and memory B cell formation are indicative of an effective and long lasting immune response generated by the vaccine and thus upon subsequent exposures could lead to antigen clearance (Fig. 5A, B, C, D). Similarly higher levels of T helper and T cytotoxic cells as well as development of memory Th and Tc were observed

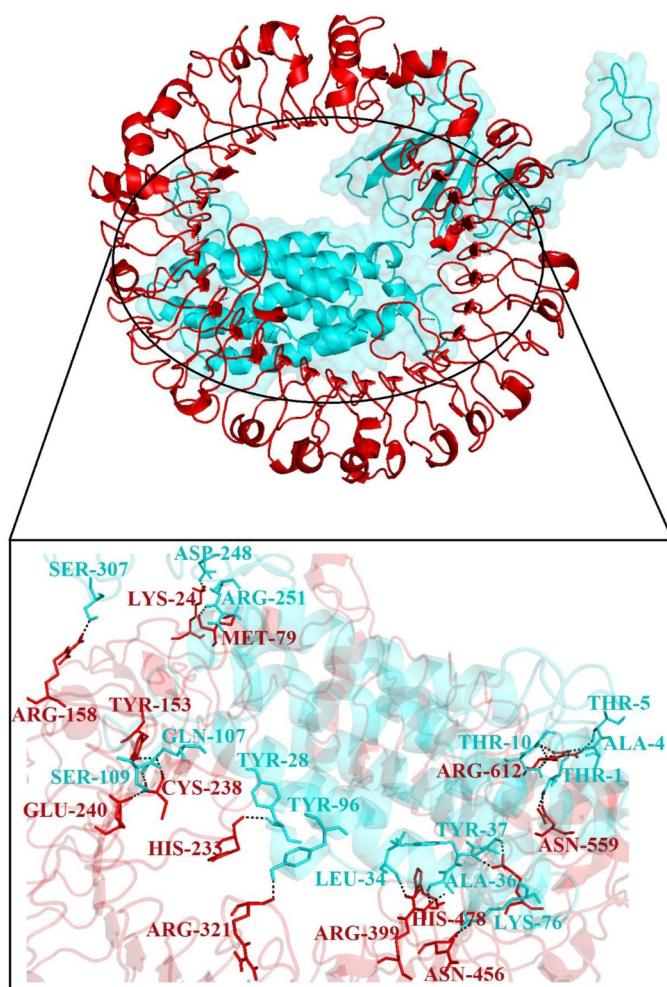


Fig. 6. Docked complex of the vaccine construct (cyan) with TLR-9 (red) in cartoon representation.

(Fig. 5E, F, G, H). The lower levels of T regulatory cells and the continuous and increased proliferation of macrophages and dendritic cells were observed during exposure to antigen (Fig. 5I, J, K). The higher levels of cytokines like IL-2, and IFN- γ were also observed, thus indicating the generation of promising antiviral response by the proposed vaccine construct (Fig. 5L).

Such multi-epitope vaccines have several unique properties which provides it an edge over single peptide based or classical vaccines like: a) it consists of B cell, CTL and HTL epitopes, thus could generate both cellular and humoral immune response in host; b) as it consists of epitopes targeting several HLAs, it can be recognized by multiple TCRs, thus could be effective in large population; c) the chances of autoimmunity or other adverse effects are reduced as the protein sequences/epitopes which overlap with human proteins and other unwanted proteins are excluded; d) several proteins can be targeted by a single vaccine, as it consists of immunogenic regions from several proteins merged as a single peptide fragment, thereby increasing the possibility of their effectiveness; e) such vaccine could provide long lasting immunity in hosts as they are also linked with an adjuvant (He et al., 2018; Lu et al., 2017; Jiang et al., 2017). Thus, to combat tumoral and viral infections, such multi-epitope vaccine could become a powerful tool in future (Zhang, 2018).

Designing of the multi-epitope vaccine using the similar kind of strategy has been carried out by different research groups against several infectious diseases like: Dengue, avian leukosis virus, Kaposi Sarcoma Virus, oncoprotein Her-2/neu, multiple myeloma, Visceral leishmaniasis etc. (Ali et al., 2017; Lu et al., 2017; Chauhan et al., 2019,

Tobias et al., 2017; Xu et al., 2015; Khatoon et al., 2017). Since the proposed vaccine consists of CTL, HTL and B cell epitopes and an adjuvant (CpG-ODNs), it has the potential to activate the host's innate as well as adaptive immune system.

3.4. Other physicochemical properties of vaccine

The vaccine construct was predicted to be immunogenic with the antigenicity score of 0.46 by VaxiJen v2.0 tool. Further, it was also found to be non-allergic by AlgPred server having the score of -0.51. Other physicochemical properties like molecular weight, Grand average of hydropathicity (GRAVY), aliphatic index, instability index, estimated half-life in human reticulocytes and theoretical pI were predicted to be 39,312.37 Da, 0.23, 83.04, 35.87, 7.2 h and 7.97 respectively. The vaccine construct was also scanned for IFN- γ epitopes. The vaccine sequence was predicted to have 6 IFN- γ epitopes (Supplementary Table 7).

3.5. Modeling of the immune receptor and analyzing its interaction with multi-epitope vaccine construct

As CMV is a ds DNA virus, its structural components will primarily be recognized by dendritic cells and B cells expressing TLR-9 on their surface. Several research groups have demonstrated the role of TLR-9 and its high expression levels at the time of CMV infection (Iversen, 2009; Paradowska et al., 2016; Tabeta et al., 2004). The CpG motifs have high affinity for TLR-9 and are well known to activate the cells expressing TLR-9 (Bode et al., 2011; Krieg, 2003). Thus, the CpG-ODNs were linked to the vaccine and served as adjuvants. The 3D model of the immune receptor-TLR-9 was modelled and refined using Raptor X and Galaxy refine servers respectively. The Ramachandran plot analysis by MolProbity revealed 98.8% residues in the allowed region (Supplementary Figure 5). The docking analysis of the constructed multi-epitope vaccine and the immune receptor TLR-9 was carried out using Cluspro. The top 10 docking outputs were taken for further analysis from multiple docking results obtained by Cluspro, in which the 4th docked complex showed the best results with the lowest energy of -1571.7 kcal/mol in terms of docking interaction pattern, indicated by forming several H-bonds between the two (Fig. 6). In addition, the binding patterns of the finally selected T cell epitopes were with the commonly occurring HLA Class I & II alleles were also analyzed by molecular docking approach (Supplementary Figure 6). All the epitopes were able to bind in the active site of HLA molecules forming number of H-bonds and with high binding affinities.

3.6. Molecular dynamics simulation

For investigating the physical movements of the multi-epitope vaccine, we performed the molecular dynamics simulations using Desmond (Schrodinger) tool. The stability and flexibility of the docked complex was analyzed for the time period of 20 ns via the root square deviation and fluctuation (RMSD and RMSF). The docked complex showed the RMSD of 7.3 Å (Fig. 7A). Only, the mild fluctuations were observed in the RMSD plot, reflecting the uninterrupted interaction between the proposed vaccine construct and the immune receptor, TLR-9. In contrast, high fluctuations, even greater than 9 Å, were observed in the RMSF plot, thus confirming the high flexibility formed between the docked complex (Fig. 7B). The secondary structure elements (SSE) of the docked complex were also observed which showed the stable confirmation throughout the simulation (Fig. 7C, D). The stable confirmation depicts the prolonged binding of the proposed vaccine construct with the immune receptor which could lead to generation of better immune response by the candidate vaccine.

The stability of the docked complex was further analysed using iMODs server. Firstly the deformability of each residue in the complex was investigated, which is represented in the form of hinges, higher the

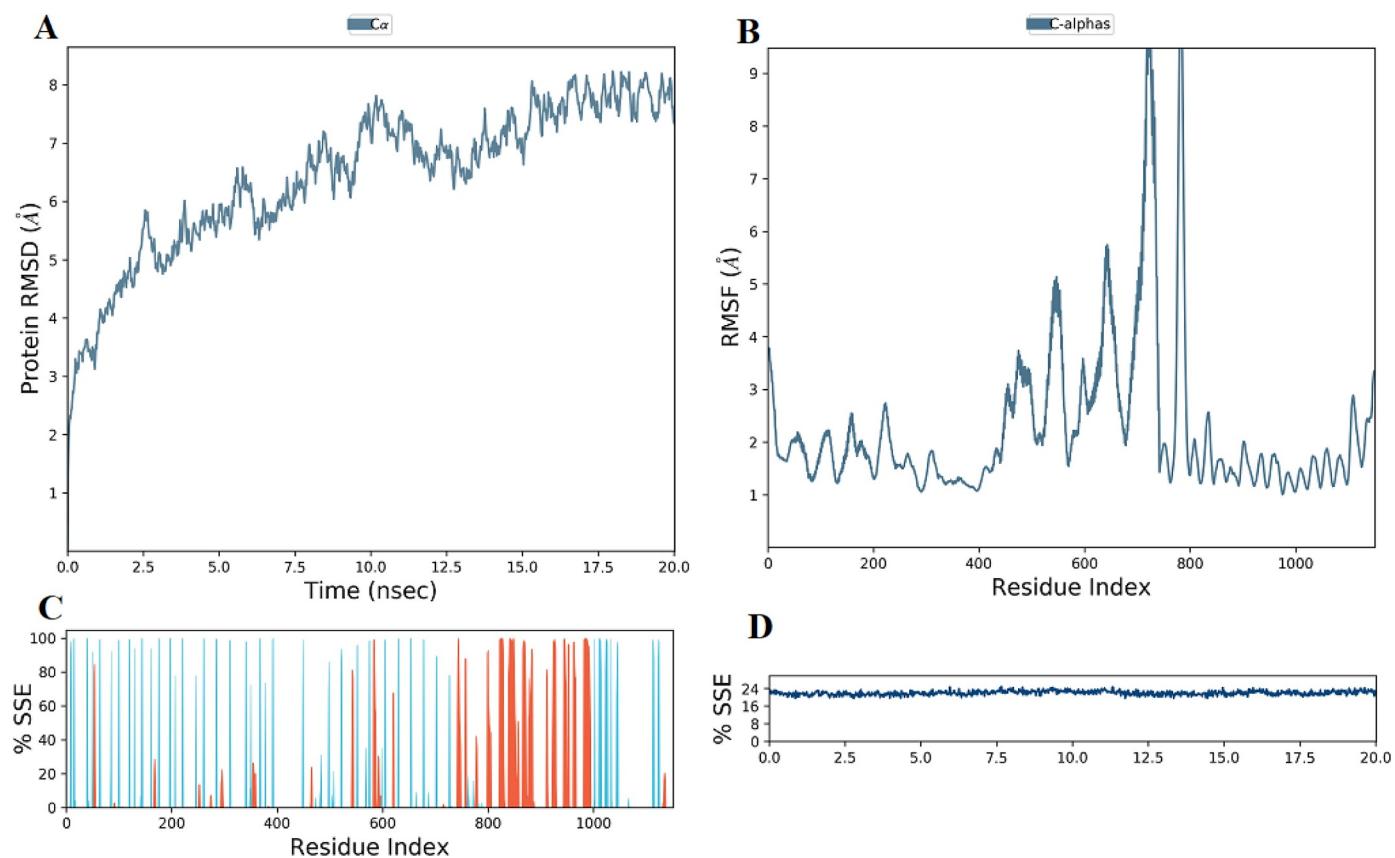


Fig. 7. Representation of the Molecular dynamics simulations of the vaccine and TLR-9 docked complex of. (A)- The RMSD plot at 20 ns. (B)- The RMSF plot (C&D)- The analysis of the Secondary structure elements (SSE) of the docked complex throughout molecular dynamics simulations.

flexibility of a particular region higher are the hinges and lower or no hinges represent the rigid regions (Fig. 8A & D). Here, we compared the deformability of the docked complex with the monomeric TLR-9 protein. The deformability analysis revealed that the distortions were significantly reduced in the docked complex as compared to the monomeric TLR-9 protein, suggesting the stabilization of the docked complex. Further, the Bfactor analysis revealed the much lesser atomic distortions in the docked complex as compared to the singlet TLR-9, thereby affirming the complex stability (Fig. 8B & E). In addition, the Eigen value of the docked complex was observed to be 8.934237e-05 and of monomeric TLR-9 was 4.979740e-06. The greater the eigen value, greater is the energy required to deform the structure. Here the docked complex showed much higher eigen value, thus indicating the stability of the complex (Fig. 8C & F). Further the coupling between the residue pair of the complex was analysed by covariance matrix analysis, where the uncorrelated, correlated and anti-correlated motions are depicted in white, red and blue colours respectively (Fig. 8G). Further the stiffness of the complex was also analysed by Elastic network analysis, where each spring is represented by a gray dot and darker the dots, greater is the stiffness of the protein (Fig. 8H). At last the variance analysis, which is inversely proportional to Eigen value was also carried out, where the cumulative (green bars) and individual (red bars) variances of the complex was analysed (Fig. 8I). The overall results of the dynamics simulations carried out in the study are in accordance to the findings and protocol followed by Kumar et al., to analyze the stability of the docked complex (Kumar et al., 2019).

3.7. Cloning and optimization

The feasibility of cloning and expression of such multi-epitope subunit vaccines in an appropriate expression vector is important. Thus, in order to analyze the cloning and expression efficiency of the vaccine

construct in an expression vector, the *in-silico* cloning was carried out. The optimization of the codon sequence was performed using Java Codon Adaptation Tool (JCat), in vecor *E. coli* (strain K12). It was composed of 1116 nucleotides, with the GC content of 58.15% and Codon Adaptation Index (CAI) 1.0 respectively. The results suggests that the proposed vaccine construct possess an efficient expression capability in *E. coli*-strain K12 (host) expression vector. Further, the adapted codon sequence of the multi-epitope vaccine was inserted into the pET28a (+) vector using SnapGene tool, for restriction cloning (Supplementary Figure 7).

4. Conclusion

HCMV is a ubiquitous pathogen causing severe complications especially in new-borns, immunocompromised patients, patients undergoing solid organ transplants, hematopoietic stem cell transplants and cancer patients. HCMV also have the ability to infect several organs and tissues in host. Moreover, the cases of drug resistance is also evolving in different parts of the world. Thus, an effective vaccine is much needed against this highly infective virus. The present immuno-informatics study was focused to identify the potential immuno-markers in the tegument protein- pp65 and other important glycoproteins which plays an important role of HCMV entry into host cells. The proteomics sequence data of the target proteins were utilized and were subjected to immunoinformatics servers in a sequential manner for prediction of epitopes. The epitopes thus predicted were passed through several immune filters designed in the study to filter out the best possible ones (Fig. 9). Thus, after application of rigorous immunoinformatics analysis, a multi epitope vaccine was constructed, consisting of epitopes (9-HTL, 8-CTL and 3-B cell epitopes), and an adjuvant (CpG-ODNs) was linked to it in order to enhance its immunogenicity. The *in-silico* simulations revealed that the vaccine is highly capable of

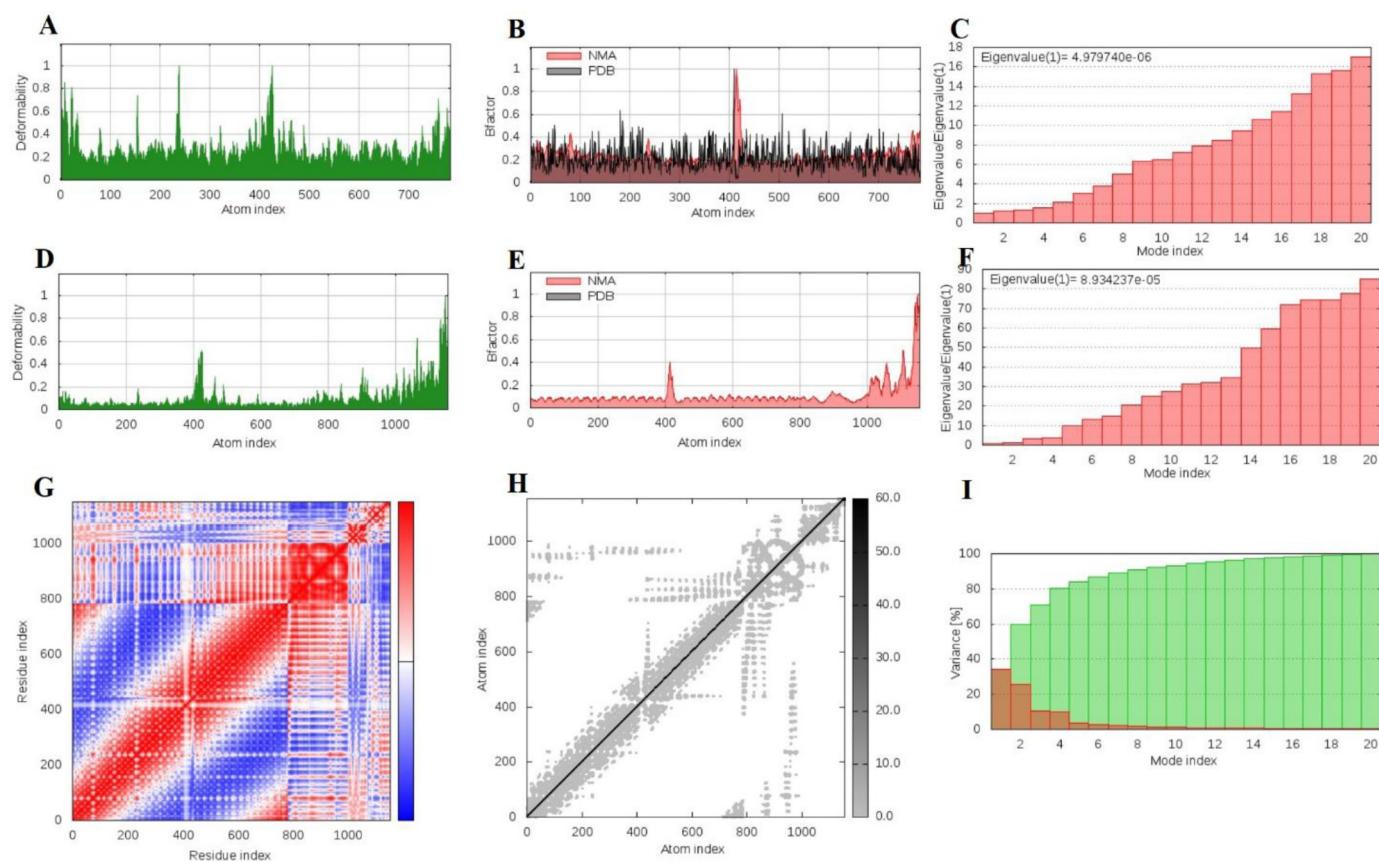
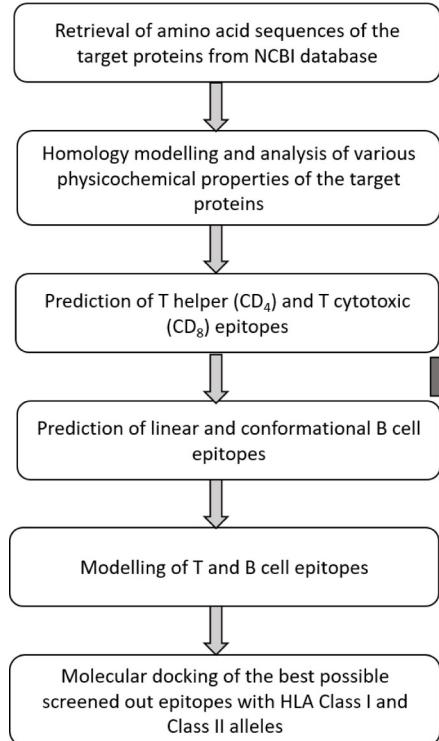


Fig. 8. iMOD simulation analysis. A to C- The Deformability, Bfactor and Eigenvalue of TLR-9; D to F- The Deformability, Bfactor and Eigenvalue of the docked complex; G- Covariance map; H- Elastic network; and I- variance analysis of the docked complex.

T AND B CELL EPITOPE PREDICTION



Immune filters applied for screening out the best possible T cell epitopes

- High affinity for HLA's
- Least IC₅₀ value
- Non-Autoimmune
- Immunogenic
- High population coverage
- 100% conserved
- Promiscuous
- Non-Allergen

Screened out CD4 and CD8 T cell epitopes

MULTI-EPITOPE VACCINE CONSTRUCTION

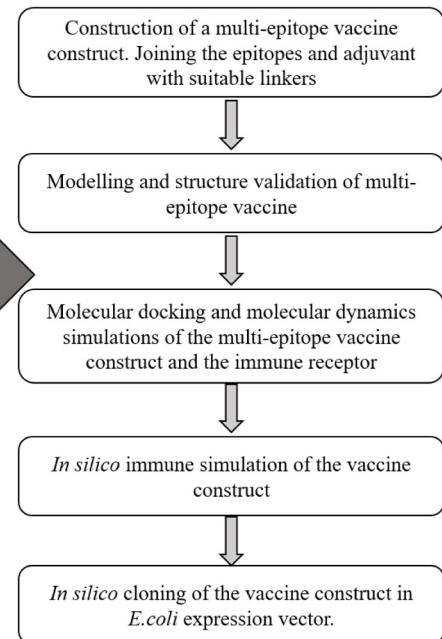


Fig. 9. Workflow for multi-epitope vaccine construction.

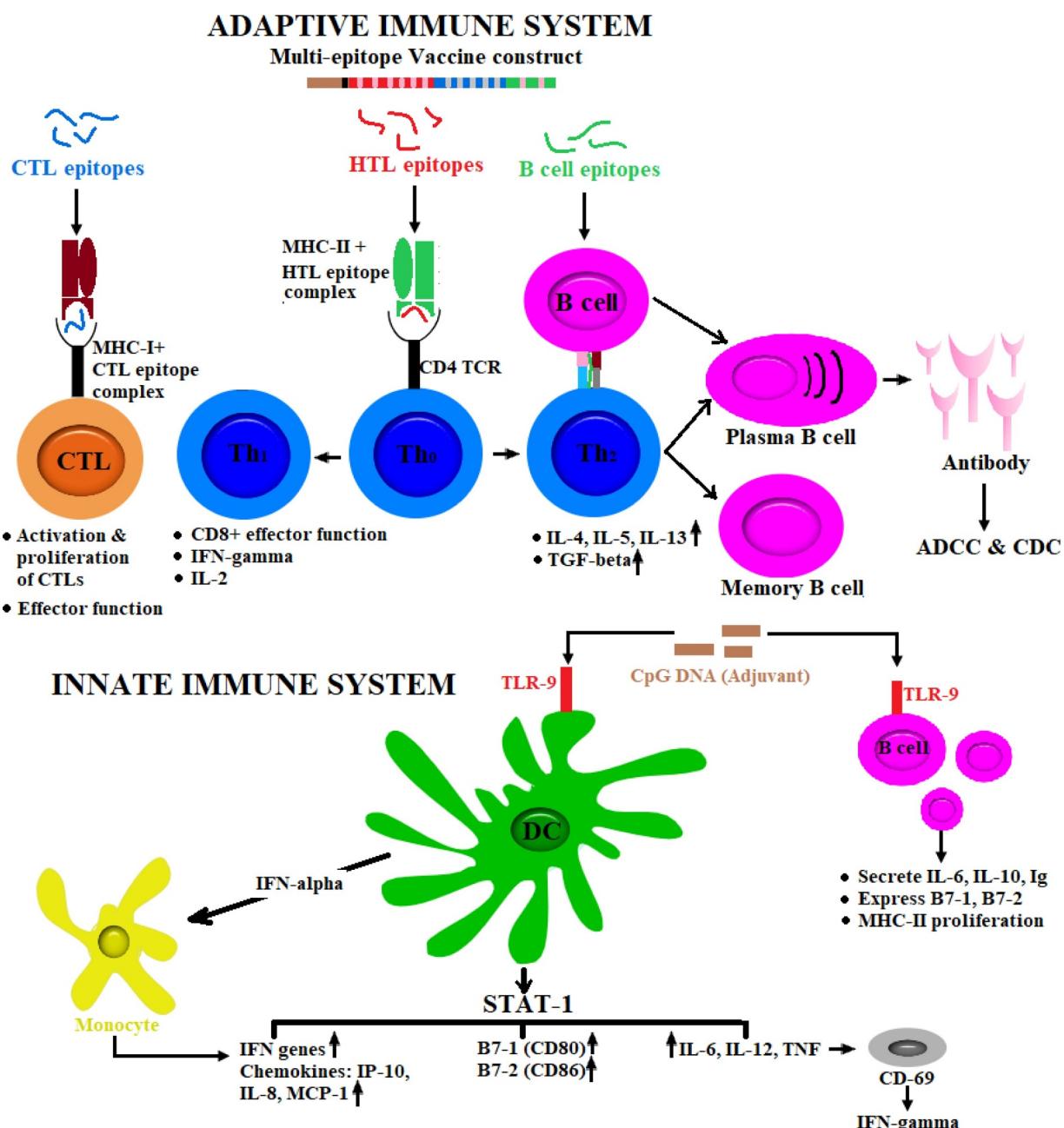


Fig. 10. Proposed immune simulation pathway in host by multi-epitope vaccine. The vaccine construct consists of a series of epitopes with an adjuvant (CpG ODNs) linked to it. The CTL (orange color) will recognize complex of CTL epitopes bounded MHC Class I molecules. This will further lead to activation and proliferation of CTLs and activation of its effector functions to kill the target cells. Similarly the MHC Class II molecules will represent the HTL epitopes to Th₀ cells which will further differentiate into Th1 and Th2 cells. Th1 cells will further stimulate cytokines and effector function of CTL cells to kill the target cells. The B cell epitopes will be recognized by B cell via B cell receptor which may further lead to Th2 cells activation. Further, the Th2 mediated cytokines and certain cell surface proteins will lead to proliferation of B cells and its differentiation into plasma and memory cells. These multi-epitope vaccine-specific antibodies secreting plasma cells will perform anti-viral activities in target cells by complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC). In addition, the TLR-9 which is expressed on the surface of antigen presenting cells will be activated on interaction with the CpG (Oligonucleotides) ODNs which may further up-regulate several immune cell types like interleukins, IP-10, IFN genes, MCP-1, CD80 and CD88 genes. Also, the CpG ODNs will also be activated indirectly certain other immune cells like T cells, NK cells, or other human monocytes. Thus, both the adaptive and innate immune system can be activated against HCMV, by the proposed vaccine construct.

stimulating different immune cell types and could provide long lasting immune response in host against HCMV. The molecular docking, molecular dynamics simulations and *in silico* cloning analysis revealed the promising results in terms of affinity, stability and ability to express in an expression vector. The proposed vaccine construct is expected to cover about 92.8% worldwide population. As the vaccine construct

contains T and B cell epitopes, it is expected to target both the adaptive and innate immune system of host (Fig. 10). The data produced in the study is carried out in the most elaborated, systematic and careful way and will definitely help the researchers in selecting the immunogenic markers in CMV for vaccine designing.

CRediT authorship contribution statement

Varun Chauhan: Formal analysis. **Mini P Singh:** Writing - review & editing.

Declaration of Competing Interest

The authors declare no conflict of interest

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.ejps.2020.105279](https://doi.org/10.1016/j.ejps.2020.105279).

References

- Alam, A., Ali, S., Ahamed, S., Malik, M.Z., Ishrat, R., 2016. From zikv genome to vaccine: in silico approach for the epitope-based peptide vaccine against zika virus envelope glycoprotein. *Immunology* (4), 386–399. <https://doi.org/10.1111/imm.12656>.
- Ali, M., Pandey, R.K., Khatoon, N., Narula, A., Mishra, A., Prajapati, V.K., 2017. Exploring dengue genome to construct a multi-epitope based subunit vaccine by utilizing immunoinformatics approach to battle against dengue infection. *Sci Rep* 7 (1), 9232. <https://doi.org/10.1038/s41598-017-09199-w>.
- Bode, C., Zhao, G., Steinhagen, F., Kinjo, T., Klinman, D.M., 2011. CpG dna as a vaccine adjuvant. *Expert Rev Vaccines* 10 (4), 499–511. <https://doi.org/10.1586/erv.10.174>.
- Britt, W., 2008. Manifestations of human cytomegalovirus infection: proposed mechanisms of acute and chronic disease. In: Human cytomegalovirus. Springer, Berlin, Heidelberg 417–470. https://doi.org/10.1007/978-3-540-77349-8_23.
- Bui, H.H., Sidney, J., Dinh, K., Southwood, S., Newman, M.J., Sette, A., 2006. Predicting population coverage of T-cell epitope-based diagnostics and vaccines. *BMC Bioinformatics* 7 (1), 153. <https://doi.org/10.1186/1471-2105-7-153>.
- Bui, H.H., Sidney, J., Li, W., Fusseder, N., Sette, A., 2007. Development of an epitope conservancy analysis tool to facilitate the design of epitope-based diagnostics and vaccines. *BMC Bioinformatics* 8 (1), 361. <https://doi.org/10.1186/1471-2105-8-361>.
- Chauhan, V., Goyal, K., Singh, M.P., 2018a. Identification of broadly reactive epitopes targeting major glycoproteins of herpes simplex virus (HSV) 1 and 2-An immunoinformatics analysis. *Infection, Genetics and Evolution* 1 (61), 24–35. <https://doi.org/10.1016/j.meegid.2018.03.004>.
- Chauhan, V., Rungta, T., Goyal, K., Singh, M.P., 2019. Designing a multi-epitope based vaccine to combat Kaposi Sarcoma utilizing immunoinformatics approach. *Sci Rep* 9, 2517. <https://doi.org/10.1038/s41598-019-39299-8>.
- Chauhan, V., Singh, M.P., Ratho, R.K., 2018b. Identification of t cell and b cell epitopes against indian HCV-genotype-3a for vaccine development-An in silico analysis. *Biologicals* 1 (53), 63–71. <https://doi.org/10.1016/j.biologicals.2018.02.003>.
- Chen, V.B., Arendall, W.B., Head, J.J., Keedy, D.A., Immormino, R.M., Kapral, G.J., Murray, L.W., Richardson, J.S., Richardson, D.C., 2010. MolProbity: all-atom structure validation for macromolecular crystallography. *Acta Crystallographica section d. Biological Crystallography* 66 (1), 12–21. <https://doi.org/10.1107/S0907444909042073>.
- Ciferri, C., Chandramouli, S., Donnarumma, D., Nikitin, P.A., Cianfrocco, M.A., Gerrein, R., Feire, A.J., Barnett, S.W., Lilja, A.E., Rappuoli, R., Norais, N., 2015. Structural and biochemical studies of hcmv gH/gL/gO and pentamer reveal mutually exclusive cell entry complexes. In: Proceedings of the National Academy of Sciences. 112. pp. 1767–1772. <https://doi.org/10.1073/pnas.1424818112>.
- Connolly, S.A., Jackson, J.O., Jarzetsky, T.S., Longnecker, R., 2011. Fusing structure and function: a structural view of the herpesvirus entry machinery. *Nature reviews Microbiology* 9 (5), 369. <https://doi.org/10.1038/nrmicro2548>.
- Dhanda, S.K., Vir, P., Raghava, G.P., 2013. Designing of interferon-gamma inducing mhce class-II binders. *Biol. Direct* 8 (1), 30. <https://doi.org/10.1186/1745-6150-8-30>.
- Doytchinova, I.A., Flower, D.R., 2007. VaxiJen: a server for prediction of protective antigens, tumour antigens and subunit vaccines. *BMC Bioinformatics* 8 (1), 4. <https://doi.org/10.1186/1471-2105-8-4>.
- EL-Manzalawy, Y., Dobbs, D., Honavar, V., 2008. Predicting linear B-cell epitopes using string kernels. *Journal of Molecular Recognition: An Interdisciplinary Journal* 21 (4), 243–255. <https://doi.org/10.1002/jmr.893>.
- Fisher, C.E., Knudsen, J.L., Lease, E.D., Jerome, K.R., Rakita, R.M., Boeckh, M., Limaye, A.P., 2017. Risk factors and outcomes of ganciclovir-resistant cytomegalovirus infection in solid organ transplant recipients. *Clinical Infectious Diseases* 65 (1), 57–63. <https://doi.org/10.1093/cid/cix259>.
- Fouts, A.E., Chan, P., Stephan, J.P., Vandlen, R., Feierbach, B., 2012. Antibodies against the gH/gL/UL128/UL130/UL131 complex comprise the majority of the anti-CMV neutralizing antibody response in cmv-hig. *J. Virol.* 24<https://doi.org/10.1128/JVI.00467-12>.
- Freed, D.C., 2013. Pentameric complex of viral glycoprotein h is the primary target for potent neutralization by a human cytomegalovirus vaccine. In: Proceedings of the National Academy of Sciences. 17. pp. E4997–E5005. <https://doi.org/10.1073/pnas.1316517110>.
- Fu, T.M., Wang, D., Freed, D.C., Tang, A., Li, F., He, X., Cole, S., Dubey, S., Finnefrock, A.C., ter Meulen, J., Shiver, J.W., 2012. Restoration of viral epithelial tropism improves immunogenicity in rabbits and rhesus macaques for a whole virion vaccine of human cytomegalovirus. *Vaccine* 30 (52), 7469–7474. <https://doi.org/10.1016/j.vaccine.2012.10.053>.
- Genini, E., Percivalle, E., Sarasini, A., Revello, M.G., Baldanti, F., Gerna, G., 2011. Serum antibody response to the gH/gL/pUL128–131 five-protein complex of human cytomegalovirus (HCMV) in primary and reactivated hcmv infections. *Journal of Clinical Virology* 52 (2). <https://doi.org/10.1016/j.jcv.2011.06.018>.
- Geourjon, C., Deleage, G., 1995. SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. *Bioinformatics* 11 (6), 681–684 PMID: 8808585.
- Grote, A., Hiller, K., Scheer, M., Münnich, R., Nörtemann, B., Hempel, D.C., Jahn, D., 2005. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. *Nucleic Acids Res.* 33 (suppl_2), W526–W531. <https://doi.org/10.1093/nar/gki376>.
- He, R., Yang, X., Liu, C., Chen, X., Wang, L., Xiao, M., Ye, J., Wu, Y., Ye, L., 2018. Efficient control of chronic lcmv infection by a CD4 t cell epitope-based heterologous prime-boost vaccination in a murine model. *Cell. Mol. Immunol.* 15 (9), 815. <https://doi.org/10.1038/emi.2017.3>.
- Heldwein, E.E., 2006. Crystal structure of glycoprotein b from herpes simplex virus 1. *Science* 313 (5784), 217–220. <https://doi.org/10.1126/science.1126548>.
- Heo, L., Park, H., Seok, C., 2013. GalaxyRefine: protein structure refinement driven by side-chain repacking. *Nucleic Acids Res.* 41 (W1), W384–W388. <https://doi.org/10.1093/nar/gkt458>.
- Iversen, A.C., 2009. A proviral role for cpg in cytomegalovirus infection. *The Journal of Immunology* 182 (9), 5672–5681. <https://doi.org/10.4049/jimmunol.0801268>.
- Jensen, K.K., Andreatta, M., Marcatili, P., Buus, S., Greenbaum, J.A., Yan, Z., Sette, A., Peters, B., Nielsen, M., 2018. Improved methods for predicting peptide binding affinity to mhc class ii molecules. *Immunology* 154, 394–406. <https://doi.org/10.1111/imm.12889>.
- Jespersen, M.C., Peters, B., Nielsen, M., Marcatili, P., 2017. BepiPred-2.0: improving sequence-based B-cell epitope prediction using conformational epitopes. *Nucleic Acids Res.* 45 (W1), W24–W29. <https://doi.org/10.1093/nar/gkx346>.
- Jiang, P., Cai, Y., Chen, J., Ye, X., Mao, S., Zhu, S., Xue, X., Chen, S., Zhang, L., 2017. Evaluation of tandem chlamydia trachomatis momp multi-epitopes vaccine in BALB/c mice model. *Vaccine* 35 (23), 3096–3103. <https://doi.org/10.1016/j.vaccine.2017.04.031>.
- Kallberg, M., Wang, H., Wang, S., Peng, J., Wang, Z., Lu, H., Xu, J., 2012. Template-based protein structure modeling using the raptorX web server. *Nat Protoc* 7 (8), 1511. <https://doi.org/10.1038/nprot.2012.085>.
- Khatoon, N., Pandey, R.K., Prajapati, V.K., 2017. Exploring leishmania secretory proteins to design b and t cell multi-epitope subunit vaccine using immunoinformatics approach. *Sci Rep* 7 (1), 8285. <https://doi.org/10.1038/s41598-017-08842-w>.
- Kim, Y., Ponomarenko, J., Zhu, Z., Tamang, D., Wang, P., Greenbaum, J., Lundsgaard, C., Sette, A., Lund, O., Bourne, P.E., Nielsen, M., 2012. Immune epitope database analysis resource. *Nucleic Acids Res.* 40 (W1), W525–W530. <https://doi.org/10.1093/nar/gks438>.
- Krieg, A.M., 2003. CpG motifs: the active ingredient in bacterial extracts? *Nat. Med.* 9 (7), 831. <https://doi.org/10.1038/nm0703-831>.
- Kumar, N., Sood, D., Sharma, N., Chandra, R., 2019. Multi-Epitope subunit vaccine to evoke immune response against acute encephalitis. *J Chem Inf Model*. <https://doi.org/10.1021/acs.jcim.9b01051>.
- Kuriata, A., Gierut, A.M., Oleniecki, T., Cierny, M.P., Kolinski, A., Kurcinski, M., Kmiecik, S., 2018. CABS-flex 2.0: a web server for fast simulations of flexibility of protein structures. *Nucleic Acids Res.* 46 (W1), W338–W343. <https://doi.org/10.1093/nar/gky356>.
- Landolfo, S., Gariglio, M., Gribaudo, G., Lembo, D., 2003. The human cytomegalovirus. *Pharmacol. Ther.* 98 (3), 269–297. [https://doi.org/10.1016/s0163-7258\(03\)00034-2](https://doi.org/10.1016/s0163-7258(03)00034-2).
- Larsen, M.V., Lundsgaard, C., Lamberth, K., Buus, S., Lund, O., Nielsen, M., 2007. Large-scale validation of methods for cytotoxic T-lymphocyte epitope prediction. *BMC Bioinformatics* 8 (1), 424. <https://doi.org/10.1186/1471-2105-8-424>.
- López-Blanco, J.R., Aliaga, J.I., Quintana-Ortí, E.S., Chacón, P., 2014. iMODS: internal coordinates normal mode analysis server. *Nucleic Acids Res.* 42 (W1), W271–W276. <https://doi.org/10.1093/nar/gku339>.
- Lu, C., Meng, S., Jin, Y., Zhang, W., Li, Z., Wang, F., Wang-Johanning, F., Wei, Y., Liu, H., Tu, H., Su, D., 2017. A novel multi-epitope vaccine from MMSA-1 and dkk 1 for multiple myeloma immunotherapy. *Br. J. Haematol.* 178 (3), 413–426. <https://doi.org/10.1111/bjh.14686>.
- Lurain, N.S., Chou, S., 2010. Antiviral drug resistance of human cytomegalovirus. *Clin. Microbiol. Rev.* 23 (4), 689–712. <https://doi.org/10.1128/CMR.00009-10>.
- Modlin, J.F., Arvin, A.M., Fast, P., Myers, M., Plotkin, S., Rabinovich, R., 2004. Vaccine development to prevent cytomegalovirus disease: report from the national vaccine advisory committee. *Clinical Infectious Diseases* 39 (2), 233–239. <https://doi.org/10.1086/421999>.
- Moise, L., McMurry, J.A., Buus, S., Frey, S., Martin, W.D., De Groot, A.S., 2009. In silico-accelerated identification of conserved and immunogenic variola/vaccinia T-cell epitopes. *Vaccine* 27 (46), 6471–6479. <https://doi.org/10.1016/j.vaccine..06.018>.
- Pance, A., 2019. How elusive can a malaria vaccine be? *Nature Reviews Microbiology*.

- Mar 17 (3), 129. <https://doi.org/10.1038/s41579-018-0148-3>.
- Pandey, R.K., Ojha, R., Aathmanathan, V.S., Krishnan, M., Prajapati, V.K., 2018. Immunoinformatics approaches to design a novel multi-epitope subunit vaccine against hiv infection. *Vaccine* 36 (17), 2262–2272. <https://doi.org/10.1016/j.vaccine.2018.03.042>. 19.
- Paradowska, E., Jabłńska, A., Studzinska, M., Skowronska, K., Suski, P., Wisniewska-Ligier, M., Woźniakowska-Gęsicka, T., Nowakowska, D., Gaj, Z., Wilczynski, J., Lesnikowski, Z.J., 2016. TLR9-1486T/C and 2848C/T SNPs are associated with human cytomegalovirus infection in infants. *PLoS ONE* 11 (4), e0154100. <https://doi.org/10.1371/journal.pone.0154100>. 22.
- Ponomarenko, J., Bui, H.H., Li, W., Fusseder, N., Bourne, P.E., Sette, A., Peters, B., 2008. ElliPro: a new structure-based tool for the prediction of antibody epitopes. *BMC Bioinformatics* 9 (1), 514. [10.1186/1471-2105-9-514](https://doi.org/10.1186/1471-2105-9-514).
- Rapin, N., Lund, O., Bernaschi, M., Castiglione, F., 2010. Computational immunology meets bioinformatics: the use of prediction tools for molecular binding in the simulation of the immune system. *PLoS ONE* 5 (4), e9862. <https://doi.org/10.1371/journal.pone.0009862>. 16.
- Ryckman, B.J., Chase, M.C., Johnson, D.C., 2008. HCMV gH/gL/UL128–131 interferes with virus entry into epithelial cells: evidence for cell type-specific receptors. In: *Proceedings of the National Academy of Sciences*. 105. pp. 14118–14123. <https://doi.org/10.1073/pnas.0804365105>. 16.
- Ryckman, B.J., Jarvis, M.A., Drummond, D.D., Nelson, J.A., Johnson, D.C., 2006. Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion. *J. Virol.* 80 (2). <https://doi.org/10.1128/JVI.80.2.710-722.2006>. 15710-22.
- Sinzger, C., Digel, M., Jahn, G., 2008. Cytomegalovirus cell tropism. in: *Human cytomegalovirus*. Springer, Berlin, Heidelberg 63–83. https://doi.org/10.1007/978-3-540-77349-8_4.
- Steinhagen, F., Kinjo, T., Bode, C., Klinman, D.M., 2011. TLR-based immune adjuvants. *Vaccine* 29 (17). <https://doi.org/10.1016/j.vaccine.2010.08.002>. 123341-55.
- Tabeta, K., Georgel, P., Janssen, E., Du, X., Hoebe, K., Crozat, K., Mudd, S., Shamel, L., Sovath, S., Goode, J., Alexopoulou, L.I., 2004. Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection. In: *Proceedings of the National Academy of Sciences*. 101. pp. 3516–3521. <https://doi.org/10.1073/pnas.0400525101>. 9.
- Tobias, J., Jasinska, J., Baier, K., Kundi, M., Ede, N., Zielinski, C., Wiedermann, U., 2017. Enhanced and long term immunogenicity of a her-2/neu multi-epitope vaccine conjugated to the carrier CRM197 in conjunction with the adjuvant montanide. *BMC Cancer* 17 (1), 118. <https://doi.org/10.1186/s12885-017-3098-7>.
- Tomar, N., De, R.K., 2018. Tools, databases, and applications of immunoinformatics. *Current trends in Bioinformatics: An Insight*. Springer, Singapore, pp. 159–174. https://doi.org/10.1007/978-981-10-7483-7_9.
- Wang, D., Shenk, T., 2005. Human cytomegalovirus UL131 open reading frame is required for epithelial cell tropism. *J. Virol.* 79 (16), 10330–10338. <https://doi.org/10.1128/JVI.79.16.10330-10338.2005>. 15.
- Wang, P., Sidney, J., Kim, Y., Sette, A., Lund, O., Nielsen, M., Peters, B., 2010. Peptide binding predictions for hla DR, dp and dq molecules. *BMC Bioinformatics* 11 (1), 568. <https://doi.org/10.1186/1471-2105-11-568>.
- Wen, Y., Monroe, J., Linton, C., Archer, J., Beard, C.W., Barnett, S.W., Palladino, G., Mason, P.W., Carfi, A., Lilja, A.E., 2014. Human cytomegalovirus gH/gL/UL128/UL130/UL131A complex elicits potentially neutralizing antibodies in mice. *Vaccine* 32 (30), 3796–3804. <https://doi.org/10.1016/j.vaccine.2014.05.004>. 24.
- Wiederstein, M., Sippl, M.J., 2007. ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Res.* 35 (suppl_2), W407–W410. <https://doi.org/10.1093/nar/gkm290>. 1.
- Wussow, F., Diamond, D.J., 2013. A vaccine based on the rhesus cytomegalovirus UL128 complex induces broadly neutralizing antibodies in rhesus macaques. *J. Virol.* 87 (3), 1322–1332. <https://doi.org/10.1128/JVI.01669-12>. 1.
- Xu, D., Zhang, Y., 2011. Improving the physical realism and structural accuracy of protein models by a two-step atomic-level energy minimization. *Biophys. J.* 101 (10). <https://doi.org/10.1016/j.bpj.2011.10.024>. 162525-34.
- Xu, Q., Ma, X., Wang, F., Li, H., Zhao, X., 2015. Evaluation of a multi-epitope subunit vaccine against avian leukosis virus subgroup j in chickens. *Virus Res.* 210 <https://doi.org/10.1016/j.virusres.2015.06.024>. 262-8.
- Zhang, L., 2018. Multi-epitope vaccines: a promising strategy against tumors and viral infections. *Cell. Mol. Immunol.* 15 (2), 182. <https://doi.org/10.1038/cmi.2017.92>.