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In-silico Designing and Testing of Multiprotein Vaccine Construct Against Cow Milk Allergy

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ABSTRACT

Cow's milk allergy is a common diagnosis in infants and children, and it manifests as a hypersensitivity reaction to the allergenic proteins in cow milk. Cow milk contains about 20 distinct protein components. The primary allergens are casein protein (alpha-s1-, alpha-s2-, beta-, and kappa-casein) and whey protein (alpha-lactalbumin and beta-lactoglobulin). There are several allergy treatments available, but they are just temporary. Developing a vaccine against milk allergy may appear utopian. Epitopes were found and exploited to build a multiprotein vaccine in this immunoinformatics study. Immunoinformatics techniques were used to predict T- and B-cell epitopes. Adjuvants were used to boost the vaccine's antigenicity. The created vaccine design was shown to be soluble, antigenic, non-allergenic, and non-toxic.

HIGHLIGHTS

- Cow milk contains around 20 proteins which may cause allergy.
- Immunoinformatics approach is used to design vaccine.
- Allergens are identified and vaccine is constructed and tested *in-silico*.

Keywords: Allergy, Epitopes, Immunoinformatics, Milk proteins, Vaccine

A sizable portion of allergies globally are caused by food allergies because of the high protein content in them. Even though data on global prevalence is generally limited, the World Allergy Organization estimated that over 250 million people, or 30% of the world's population, suffer from allergies (Pawankar, 2014). Allergy, intolerance, and hypersensitivity are terms that are frequently used interchangeably. In the developed world, prevalence of milk allergy in newborn is around 2-3% (Lifschitz and Szajewska, 2015; Edwards and Younus, 2022). In milk allergy condition, the body's immune system reacts to a particular milk protein, which sets off an immunological reaction and an attempt to neutralize the offending protein. When the body comes into contact with the protein again, the immune system recognises it and starts an attack, releasing histamine and other immune mediators as part of the attack. The symptoms and indicators of a cow's milk allergy are brought on by this chemical release.

There are almost 20 different protein fractions in cow milk. Casein protein (alpha-s1-, alpha-s2-, beta-, and kappa-casein) and whey protein (alpha-lactalbumin and beta-lactoglobulin) are the main allergens (Wood *et al.,*

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2013). Most people who are allergic to cow milk are also sensitive to casein and whey proteins (Bartuzi *et al.,* 2017). Cow's milk allergy is most usually brought on by a non-IgE mediated pathway. There are two more subtypes of cow's milk allergy reactions: rapid onset, typically IgE mediated, when symptoms appear within an hour of consumption, and gradual onset, non-IgE mediated, where symptoms appear hours or days after consumption (Kansu *et al.,* 2016). Urticaria/hives, wheezing, itching or a tingling sensation around the mouth or lips, angioedema, coughing or shortness of breath, vomiting, and anaphylaxis are just a few of the symptoms that might appear suddenly. Symptoms with a slow onset include colic, hematochezia, diarrhoea, and stomach pains (Luyt *et al.,* 2014). Finding advantageous polymorphisms in the immunogenicity-related genes and exploiting them to breed milk production free of allergy is the only long-term cure for milk allergy. It might seem idealistic to develop a vaccine for milk allergy given these circumstances.

There is a very thin line separating immunotherapy from vaccinations, though. Interferons, cytokines, such as Granulocyte-macrophage colony-stimulating factor, and other growth factors are used in immunotherapy, a non-vaccine treatment, to alter the immune response (Dalgleish *et al.,* 2015). Adjuvants, which are often included in vaccinations to direct or increase a specific immune response, can be used to improve immunotherapies. In animal models, adjuvants that support Th1-polarized and regulatory immune responses, while repressing Th2 immunity are the most promising. When added to AIT, adjuvants may lengthen immune responses and reduce the amount and frequency of allergens required to elicit therapeutic efficacy (Landers and O'Konek, 2021). In this way, immunotherapy, which is typically a passive method of managing food allergies, can be transformed into vaccinations, a method that is active. Prioritizing proteins to find B and T cell epitopes is also required for the design of vaccines that are either directed against a pathogen that causes disease or for a food allergy. So, in this case, we tried to identify epitopes and incorporate them into the in silico vaccine design.

MATERIAL AND METHODS

Sequences Retrieval

The protein sequences of Alpha-lactalbumin (Uniprot Id: P00711), Beta-lactoglobulin (Uniprot Id: P02754), Alpha-S1-casein (Uniprot Id: P02662), Alpha-S2-casein (Uniprot Id: P02663), Beta-casein (Uniprot Id: P02666) and Kappa-casein (Uniprot Id: P07498) (hereafter referred as AL, BL, CAS1, CAS2, BC and KC, respectively) were downloaded from Uniprot data bank (https://www.uniprot.org/uniprotkb) to identify potential epitopes and for further vaccine construction. The physiochemical characteristics of the proteins were determined using the ProtParam tool of the ExPASy database server (Gasteiger *et al.,* 2005). The antigenicity of the proteins was determined using the VaxiJen server (Doytchinova *et al.*, 2007). The allergenicity of the proteins was examined using AllerTOPv2.0 (Dimitrov *et al.,* 2014).

Designing of Multi-Protein Multi-Epitope Vaccine Construct (MPVC)

The chosen HTL, CTL, and B-cell epitopes of five proteins were connected using GPGPG, AAY, EAAAK, and KK linkers, respectively, to create a multiprotein multi-epitope (MPVC) vaccine construct. Four adjuvants were also included into the vaccine design using linkers: defensin, universal memory T-cell helper peptide (TpD), PADRE (Pan HLA-DR reactive epitope), and an M-cell ligand. Defensin was added to the N terminal to increase immunogenicity, while M-cell ligand was added to the C terminal, followed by the inclusion of HHHHHH to make it easier to purify the vaccine in future studies. Chauhan *et al.* (2019)'s technique was used to create an MPVC vaccine against an allergy to milk proteins with the following criteria: (a) be promiscuous; (b) overlapping CTL and HTL epitopes; (c) be immunogenic; (d) have a strong affinity for HLA alleles; and (e) not overlap with any human gene.

Antigenicity, Allergenicity, and Physiochemical Properties of MPVC Vaccine Construct

The vaccine's antigenicity was evaluated using the VaxiJen server (Doytchinova *et al.*, 2007) and AllerTOPv2.0 (Dimitrov *et al.,* 2014). The physiochemical properties of the vaccine were determined by employing the ProtParam tool of the ExPASy database service (Gasteiger *et al.,* 2005).

Structure Prediction, Validation, and Docking of Vaccine Construct with the Receptor

Phyre2 server was used to predict the tertiary structure of the vaccine construct, whereas PSIPred 4.0 Protein Sequence Analysis Workbench (Geourjon and Deleage, 1995) was used to predict the secondary structure (Kelley *et al.,* 2015). Web servers from PROCHECK v. 3.5 (Laskowski *et al.,* 2006) and ProSA (Wiederstein and Sippl, 2007) were used to validate the vaccine build model with the highest TM score. The Cluspro v. 2 (Kozakov *et al.,* 2017) protein–protein docking web server was used to dock vaccine receptors to establish the vaccination's affinity for the TLR3 receptor (PDB ID: 2A0Z) and TLR4 receptor (PDB ID: 3FXI). C-ImmSim server (Rapin *et al.,* 2010) was used to describe the real-world immunogenic profiles and immunological response of the MPVC vaccination.

RESULTS AND DISCUSSION

Immunotherapy is regarded to be the most effective method of treating and relieving allergy symptoms (CDCP, 2020). In this study, we investigated a reverse vaccinology strategy for generating a milk protein allergy vaccine with numerous epitopes that may successfully elicit humoral and cellular mediated immune responses.

Physiochemical Properties of Proteins used for Vaccine Construction

Among six proteins under study (Table 1), AL had lower amino acid residues of 142 and BC had a maximum of 224 amino acid residues. Out of all six proteins, except AL, all were predicted as unstable in the instability index and in antigenicity index AL, BC and KC did not qualify as antigen as it got a score less than threshold of 0.4 set by the VaxiJen server. Further, AllerTOPv2.0 webtool predicted all proteins as allergens, except KC (Table 1).

T Cell and B cell epitope prediction

The CTL epitopes were predicted for all the proteins using the NetCTL1.2 server and evaluated for their antigenicity by the VaxiJen server. For the AL, the server has predicted 134 probable CTL epitopes, out of which 2 epitopes crossed the threshold set by the prediction model of NetCTL1.2 server. For BL, 3 epitopes out of 170; for CAS1, 2 out of 206; for CAS2, 8 out of 215; for BC, 4 out of 216, and for KC, 3 out of 174 epitopes exceeded threshold value and hence were considered as potential

Table 1: Physiochemical properties, allergenicity and antigenicity of proteins used for vaccine construction

Properties	Alpha-lactalbumin	Beta-lactoglobulin	Alpha-S1-casein	Alpha-S2-casein	Beta-casein	Kappa-casein
Number of amino acids	142	178	214	222	224	182
Molecular weight (KDa)	16.246	19.883	24.528	26.018	25.107	20.305
Asp + Glu	20	26	32	28	23	11
Arg + Lys	13	19	21	31	16	14
Instability index	27.58 (Stable)	40.12 (Unstable)	56.03 (Unstable)	44.68 (Unstable)	94.12 (Unstable)	56.48 (Unstable)
Aliphatic index	91.27	106.40	85.19	73.74	97.37	83.57
Grand av. of hydropathicity (GRAVY)	-0.169	-0.010	-0.481	-0.704	-0.154	-0.263
Allergenicity (AllerTOPv2.0)	Allergen	Allergen	Allergen	Allergen	Allergen	Non-Allergen
Nearest protein (AllerTOPv2.0)	NCBI gi number 163283	NCBI gi number 125910	NCBI gi number 92	NCBI gi number 162929	UniProtKB accession number P02666	UniProtKB accession number P07498
Antigenicity (VaxiJen threshold: 0.4)	0.3060 (Probable Non-Antigen)	0.5018 (Probable Antigen)	0.4039 (Probable Antigen)	0.4488 (Probable Antigen)	0.3871 (Probable Non-Antigen)	0.5280 (Probable Antigen)

epitopes. But in order to reduce the length of vaccine construct top two epitopes from each of the proteins were considered (Table 2). Using the IEDB MHC II server, HTL epitopes, a crucial component of the adaptive immune response, were predicted, out of which one potential epitope from each of the proteins was included in vaccine construct (Table 3). The web server ABCpred was utilized to anticipate B-cell epitopes and listed in Table 4. MHC class II binding peptides are generally 12-25 amino acids long, whereas MHC class I binding peptides are 8-11 amino acids long. According to the study's predictions and evaluations of T cell epitopes based on qualities such as antigenicity, allergenicity, immunogenicity, and toxicity, the chosen T-cell epitopes looked to have high scores for these attributes. Furthermore, B-cell epitopes were classified into two types: discontinuous or conformational B-cell epitopes and continuous or linear B-cell epitopes. Antigenicity, allergenicity, and toxicity assessments were performed on the projected linear B-cell epitopes with higher cut-off values (0.8 and above), and the epitopes with the highest scores were chosen for MPVC.

Table 2: CTL epitopes used for MPVC vaccine construct for milk allergy

Protein	Epitopes	C terminal Amino acid number	NetCTL Score
Alpha-lactalbumin	ILDKVGINY	114	2.5805
	CTTFHTSGY	47	2.5613
Beta-lactoglobulin	VLDTDYKKY	110	1.9230
	DTDYKKYLL	112	1.1582
Alpha-S1-casein	YPELFRQFY	161	0.7987
	AYPSGAWYY	173	0.7928
Alpha-S2-casein	ALNEINQFY	96	2.0147
	ESIISQETY	27	1.3767
Beta-casein	LTDVENLHL	142	2.6530
	FAQTQSLVY	67	2.4034
Kappa-casein	KTAPYVPMY	41	2.7687
	YVPNSYPYY	50	1.2687

Table 3: HTL epitopes used for MPVC vaccine construct for milk allergy

Protein	Start	End	Peptide	Adjusted rank	Epitope core
Alpha-lactalbumin	1	15	MMSFVSLLLV GILFH	4.9	FVSLL LVGI
Beta-lactoglobulin	34	48	TWYSLAMA ASDISLL	4.8	YSLAM AASD
Alpha-S1-casein	164	178	LFRQFYQL DAYPSGA	1.6	FYQLD AYPS
Alpha-S2-casein	3	17	FFIFTCLLAVA LAKN	0.16	FTCLL AVAL
Beta-casein	2	16	KVLILACLVAL ALAR	0.01	LACLV ALAL
Kappa-casein	2	16	KSFLLVVNA LALTLP	0.01	FLLVV NALA

Table 4: B cell epitopes used for MPVC vaccine construct for milk allergy

Protein	Sequence	Start	Score
Alpha-lactalbumin	KVGINYWLA HKALCSE	117	0.09
Beta-lactoglobulin	QCLVRTPEVD DEALEK	136	0.84
Alpha-S1-casein	AESISSSEEI VPNSVE	77	0.88
Alpha-S2-casein	EESIISQETY KQEKNM	26	0.96
Beta-casein	PGEIVESLS SSEESIT	24	0.87
Kappa-casein	AIAINNPYV PRTYYAN	68	0.88

Designing of Multi-Protein Multi-Epitope Vaccine Construct

For inclusion in the MPVC, the highly antigenic 6 HTL and 12 CTL epitopes with the greatest affinity for the HLA alleles as well as 6 B-cell epitopes with non-allergenic, non-toxic, and immunogenic properties were chosen. Following ~~the~~ coupling of the adjuvant defensin with the B cell epitope at the N terminal using the EAAAK linker, the B cell epitopes, CTL epitopes, and B HTL epitopes were connected using the AAY, GPGPG, and KK linkers, respectively. Using EAAAK linkers, adjuvants such as PADRE (Pan HLA-DR reactive epitope), Universal memory T-cell helper peptide (TpD), and M cell ligand

were coupled into the vaccine design. To make it simple to purify the vaccine, HHHHHH and EAAAK linker were connected at the C terminal (Fig. 1). The different adjuvants that were added to MPVC were to increase innate and adaptive immune responses as well as MPVC passage over the intestinal membrane barrier. The vaccine's antigenic, allergic, and physiochemical qualities were then confirmed. The construct displayed high antigenicity while simultaneously being non-allergic and non-toxic.

Physiochemical properties, antigenicity, and allergenicity of multi-epitope vaccine construct

The Protparam tool was used to determine the physiochemical characteristics of the MPVC. The final MPVC had a molecular weight of 66 KDa with 613 amino acid residues, 51 negatively charged residues (Asp + Glu), and 59 positively charged residues (Arg + Lys). The construct's stability index was 37.03 indicating the constructed vaccine as stable protein. Grand average of hydropathicity (GRAVY) and the aliphatic index were determined to be 82.1 and -0.143, respectively. The computed value of the theoretical isoelectric point (PI) was 8.58. The developed vaccine was discovered to be antigenic in nature when the vaccine build sequence was examined in the VaxiJen server, with an overall prediction score of 0.6253. Additionally, this vaccine design was proved to be non-allergenic, and AllerTOPv2.0's predicted closest protein, with the UniProtKB accession number Q8IWY9, was found.

Secondary and tertiary structure prediction and validation

The SOPMA server was used to analyze the secondary structure of vaccine construct, which showed that the secondary structure consisted of 42.41% (260 amino acid residues) helix, a 16.15% (99 amino acid residues) extended strand, 4.89% (30 amino acid residues) turn coil, and 36.54% (224) random coil (Fig. 2). The Phyre2 webtool and Galaxy Refine predicted and improved the MPVC's tertiary structure (Fig. 3A). The GDT-HA, RMSD, and MolProbity scores for the top-performing model were 0.9389, 0.480, and 2.156, respectively. The stereochemical quality was checked using PROCHECK, and Ramachandran plot analysis of the modelled structure showed that 76.3% of the residues were in the regions that were most favoured, 13.2% were in the additional allowed regions, 10.5% were in the generously allowed region, and 0.0% were in the disallowed region (Fig. 3B). A ProSA webtool was employed to check for probable faults in the protein 3D model, and it projected a negative Z-score of -4.75, indicating the model's high quality (Fig. 3C). These findings supported the projected model's accuracy.

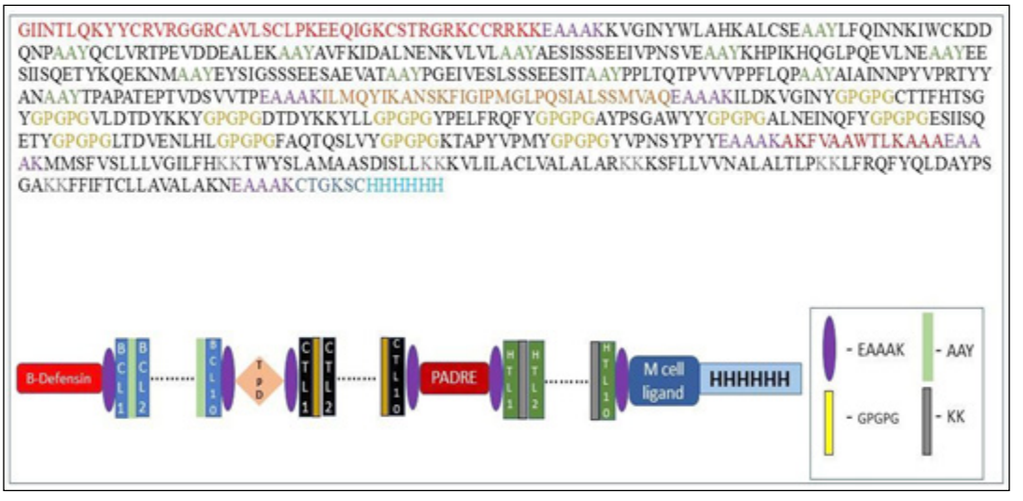

Fig. 1: Multiprotein multi-epitope vaccine construct with different linkers and adjuvants. Different sequences of vaccine are color coded and diagrammatically represented

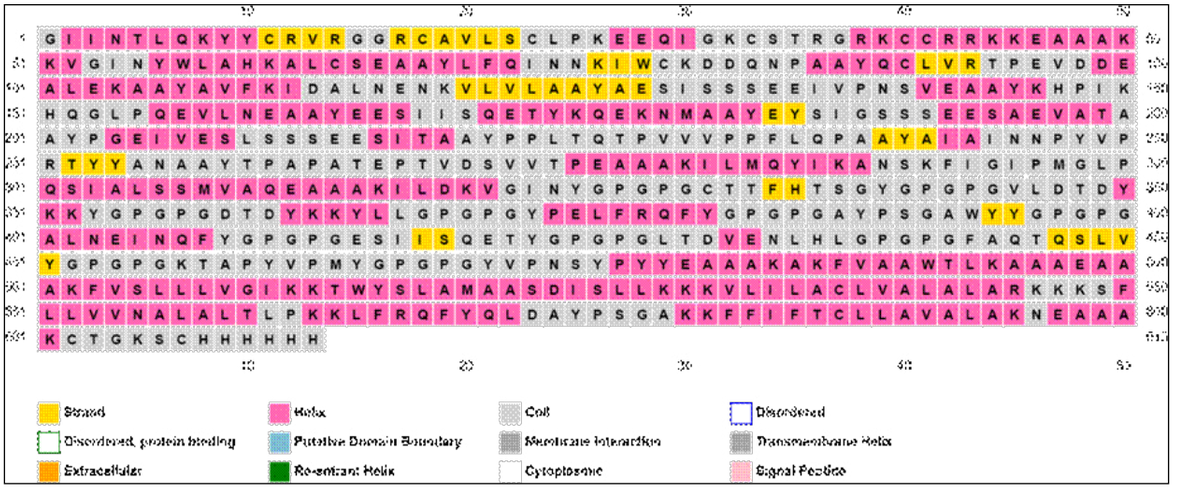

Fig. 2: Secondary structure of vaccine construct

Table 5: Docking results with binding energy

Macromolecule for docking	Epitope	Best model	Representative	Weighted score
TLR3	Merged CTL epitopes	Model 0	Center	-517.6
			Lowest Energy	-586.7
	Merged B cell epitopes	Model 0	Center	-1040.4
			Lowest Energy	-1168.9
	Merged HTL epitopes	Model 0	Center	-517.9
			Lowest Energy	-623.8
TLR4	Merged CTL epitopes	Model 0	Center	-1036.8
			Lowest Energy	-1036.8
	Merged B cell epitopes	Model 0	Center	-999.1
			Lowest Energy	-1391.5
	Merged HTL epitopes	Model 0	Center	-1019
			Lowest Energy	-1206

Docking of multi-epitope vaccine construct with receptors

From the protein data repository, the 3D structures of human TLR3 and TLR4 were obtained (PDB ID: 2A0Z and 3FXI). The ClusPro version 2 protein–protein docking server was used to perform the molecular docking study. Before docking, CTL, HTL and B cell epitopes were constructed separately in order to increase accuracy of the model. With their associated cluster scores, Cluspro version 2 predicted 10 models each for the particular epitope group-TLR4 complex and particular epitope group-TLR3 complex. Models with lowest energy were chosen as the best-docked complex among these models (Table 5). This denotes the possibility of a molecular interaction between the anticipated vaccine design and TLR3 and TLR4 receptors. Docking studies on the molecular interactions between vaccines and TLR3 and TLR4 found that the produced vaccine has a great affinity for the toll-like receptors to recognize pathogen molecular patterns and initiate the immune response. The adjuvant defensin in the current MPVC, as a TLR agonist, can interact with a range

of TLRs to boost both innate and adaptive immunity. As a consequence, the MPVC in combination with the defensin adjuvant has the potential to elicit an immune response that is useful in the treatment of milk allergy.

Immune simulations of vaccine construct

The final vaccine construct's ability to elicit an immunological response was examined using the C-ImmSim simulator. Three events are the main emphasis of the whole simulation: the binding of B-cell epitopes, the binding of HLA Class I and II epitopes, and the binding of the TCR, in which the interaction of the HLA peptide complex should be shown. The combined findings of the immune reactions following three antigen exposures showed that there was an increased primary immune response to the antigenic fragments, which was demonstrated by the steady rise in IgM level following each antigen exposure. Similar to the first reaction, the secondary response was distinguished by the sufficient production of IgM + IgG rather than IgM. Additionally, it was shown that IgG1 + IgG2 and IgG1 levels had risen (Fig. 4).

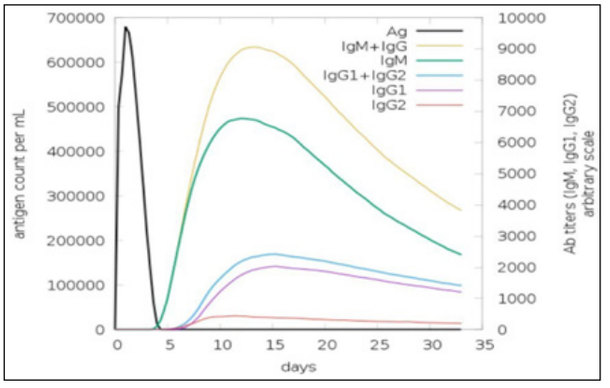

Fig. 4: Vaccine response in inducing immunoglobulins

A drop in antigen levels was seen when the vaccine was administered again, showing the emergence of an immunogenic response in the form of immunological memory. The accurate clonal proliferation of the B-cell and T-cell populations is supported by the higher levels of all circulating immunoglobulins. Additionally, a rise in the B-cell population was associated with an increase in immunoglobulin expression, which led to a drop in antigen concentration. Additionally, the number of Th (helper) and Tc (cytotoxic) cells increased steadily as memory development progressed. Dendritic cells, macrophages, and total NK cells were also increased. Additionally, it was noted that vaccine boosted IFN-gamma production. These findings demonstrated that the MPVC suggested in this work had the potential to elicit a potent immunological response, one that would persist even after repeated exposure. The consistent increase in IgG subclass levels as well as immunological cells (B and T cell population) in C-ImmSim indicated that humoral immunity had been activated. Because allergen-specific IgG antibodies appear to play a substantial role in blocking allergic immune responses, passive immunization using allergen-specific IgG antibodies has emerged as another approach for allergy vaccination (Flicker *et al.,* 2011). If more potential epitopes from other proteins are incorporated, the MPVC may suffer from a restriction of the construct's complexity in addition to synthesis issues (Srinivasan *et al.,* 2022).

With evidence indicating that immunotherapy can modify allergen-specific immune responses while encouraging desensitization, this method is widely recognised as a potential treatment option for food allergies (Ismail and Tang, 2012). Immunotherapies have been shown to be beneficial in the treatment of egg allergy; for example, modified ovalbumin containing mannose or glucomannan might be useful (Rupa and Mine, 2014). The six milk proteins employed in the current study's MPVC vaccine must be tested for oral vaccination as an active immunotherapy technique. Because it is developed generically, this vaccine, if evaluated *in vivo* and followed by clinical trials, has the potential to protect a substantial section of the population from milk allergy.

CONCLUSION

The existence of all three types of epitopes, including CTL, HTL, and B-cell epitopes, makes the current study advantageous in terms of giving a possible MPVC. The vaccine construct developed was discovered to be soluble, antigenic, non-allergenic, and non-toxic. Overall, the purpose of this in silico work is to highlight the potential of immunoinformatics to target proteins and to suggest an unique MPVC for offering fresh rays of hope in the early stages of vaccine development and subsequent experimental validation to give protection against milk allergy. Overall, our findings call for and should allow

more thorough research verifying this new construct's *in vivo* immune response and elucidating their underlying cellular/molecular pathways for future therapeutic applications.

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