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Effect of oral administration of in silico epitope-based *SARS-CoV-2 virus* with ISCOM adjuvants on increasing the number of NK cells and serum IgG in mice

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ABSTRACT

Background: Vaccines are one of the best solutions to deal with the COVID-19 pandemic. Epitope vaccines can be searched in silico. The selection of in silico epitope-based *SARS-CoV-2* which is used as a vaccine candidate must be able to trigger an immune response, such as proteins from the spike (S), envelope (E), membrane (M) in *SARS-CoV-2*. This study aims to determine the potential for in silico epitope-based *SARS-CoV-2* from S, EM, and SEM which is immunogenic, non-toxic, and non-allergenic. And evaluate the immune response by measuring the number of NK cells in the spleen and serum IgG levels in mice.

Method: This research was carried out in 2 stages, an in silico exploratory study and an experimental study. The exploratory stage consisted of selecting immunogenic, non-toxic, non-allergic vaccine candidates, molecular docking tests, and epitope conjugation with an adjuvant in the form of ISCOM which was observed with a TEM microscope. The first group was the control, and the second group was given ISCOM. The remaining groups were each given the S, EM, and SEM epitope which had been conjugated with ISCOM and all were given orally. In 5 groups, NK cell levels were measured using a flow cytometer, while IgG levels were measured using Elisa.

Research: The results of the in-silico test showed that 3 epitopes of S (FLVLLPLVSSQCVN), E (VNSVLLFLAFVVFLLVTLASS), and M (LYIIKLIFLWLLWPVTLACFV-LAAVY) were immunogenic, non-toxic, and non-allergic. Oral administration of in silico epitope-based SARS-CoV-2 in mice could increase the highest number of NK cells in the administration of S epitope. Meanwhile, the highest serum IgG level was given with the combination of SEM epitope.

Conclusion: Oral administration of an in-silico epitope based on *SARS-CoV-2* from spike, envelope, and membrane can increase the number of NK cells in the spleen and IgG levels in mice.

Keywords: epitope, in silico, IgG, NK cells, *SARS-CoV-2*.
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BACKGROUND

WHO announced that the COVID-19 outbreak had been declared a pandemic on January 30, 2020. Vaccines are one of the best solutions to overcome the COVID-19 pandemic. There are several reasons why vaccines are still being developed today, one of which is the structure of the vaccine. Peptide vaccines have advantages in terms of disease specificity, purity, production capacity, and production cost efficiency. Based on this, efforts are needed to develop a peptide-based COVID-19 vaccine.[1]

An important parameter of vaccine design is to ensure that: the vaccine made is immunogenic to induce humoral and/or cellular immune responses against the targeted virus, is non-toxic and non-allergenic so it is safe to use.[2]

In addition, this research was conducted because we wanted to know the immunological potential of the results of an in-silico study based on The Immune Epitope Database Analysis Resource (IEDB), which further exploration results in research are expected to open up new dimensions in compiling an epitope-based vaccine regimen from the SARS-CoV-virus. 2 based on in-silico exploration which was continued to experimental animals.

Both innate and adaptive immune responses are essential for controlling viral infections. IgG strength and duration after infection are the keys to immunity from SARS-CoV-2. NK cells also interact with dendritic cells and play a part in the process of antigen presentation and adaptive immunity against SARS-CoV-2. The number of NK cells also plays an important role in IgG immunity in COVID-19. The binding of CD16 to NK cells by virus-infected antibody-coated cells results in antibody-dependent cellular cytotoxicity.[3] Therefore, this study aims to determine the potential for in silico epitope-based SARS-CoV-2 from S, EM,

and SEM which is immunogenic, non-toxic, and non-allergenic. And evaluate the immune response by measuring the number of NK cells in the spleen and serum IgG levels in mice.

MATERIALS AND METHODS

Identification and selection In-Silico Epitope-Based SARS-CoV-2 Virus (SARS-CoV-2 spike glycoprotein, SARS-CoV-2 envelope, and SARS-CoV-2 membrane)

Selection of virulent protein for SARS-CoV-2 virus using data from NCBI (https://www.ncbi.nlm.nih.gov/nuccore/MN996531.1/) and alignment using the Basic Local Alignment Protein Search Tool (BLAST). and Bioedit. Prediction of MHC gene loci including HLA-I and HLA-II using Immune Peptide Database and Analysis Resource (IEDB) and Analysis of antigenic proteins identified using Kolaskar and Tongaonkar Antigenicity software from IEDB.

Vaccine antigenicity was assessed using the antigenic peptides prediction tools program accessed at (http://imed.med.ucm.es/Tools/antigenic.pl) proteins with a probability score as antigen greater than 0.8 were considered for vaccine manufacture. Prediction of Tertiary Structure of protein using PyMOL software. The molecular docking process uses Autodock Vina, PyMOL, and Protein Plus software.[2]

Analysis Antigenicity, Toxicity and Allergenicity Test

Vaccine antigenicity was assessed using the antigenic peptides prediction tools program accessed at (http://imed.med.ucm.es/Tools/antigenic.pl) proteins with a probability score as antigen greater than 0.8 were considered for vaccine manufacture. Immunogenicity of vaccine candidates was measured using the VaxiJen Server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) (Doytchinova and Flower 2007b) and the ANTIGENpro module of the SCRATCH protein predictor (http://scratch.proteomics). ics.uci.edu/).[4] Toxicity prediction of vaccine candidate is done through SVM Score with ToxinPred software (http://crdd.osdd.net/raghava/toxinpred/).[5,6] Allergenicity tests were carried out using an algorithm implemented in a specially designed Web site, named AllerTOP v2.0. The algorithm is freely accessible on the website https://www.ddg-pharmfac.net/AllerTOP/.[7]

Based on the results of in silico exploration in our next study, a custom order will be made from Bankpeptide Biological Technology Co., LTD to be processed into peptides according to the epitope sequence that has been found in silico so that it can be conjugated with ISCOM adjuvants.

Immunogenicity Test in-silico Epitope-based SARS-CoV-2 in mice

Epitope SARS-CoV-2 weighed 10 mg and later dissolved in 5 ml of PBS (0.2 M pH 7.4) and homogenized with vortex. Next 10 mg of Quill A saponin was added (solution A) and homogenized with vortex. Mixture peptide and solution A which has been homogeneous then added 200 L solution B (1% dissolved phosphatidylcholine in 20% egg yolk lecithin and 1% cholesterol) then homogenized with vortex. Next mixture of the dialysis in 0.2 M PBS solution pH 7.4 for 2-3 hours at space. Next solution dialysis was replaced and dialyzed back at 4 ° C overnight. The dialysate (dialysis result) was then centrifuged at 10,000 g at 4 ° C for 5 minutes. The pellets resulting from the centrifugation process were resuspended with 25% sucrose which had been dissolved in 0.2 M PBS solution pH 7.4 (1:1 ratio). Suspension of the centrifuged use ultracentrifuge with 257,000 g at 4° C for 2 hours. supernatant was moved to another tube while pellets were added with 2.5 ml of PBS. Conducted checking resuspension pellet and supernatant with nanodrop and if result positive, then confirmed with TEM (Transmission Electron Microscopy) microscope.

We use mice (*Mus musculus*) strain Balb /C with a total of 25 mice (n=25) divided into 5 groups the treatment is given orally through a probe. P1 (group Control) was given 100µl PBS, P2 (ISCOM group) was given ISCOM 30 g/100 L, P3 (ISCOM group) *epitope* S) was given ISCOM - conjugated spike *epitope* with dose of 30 g/100 l, P4 (Group *epitope EM*) is given *ISCOM* - conjugated envelope and membrane epitope with dose 30 g/100 L, P5 (Group *SEM epitope*) is given *ISCOM* - conjugated spike, envelope and membrane epitope with dose of 30 g/100 L. We give treatment on the week first, followed by 3x booster given every week for 28 days.

Flow cytometry examination

Measurement of the number of NK cells using spleen samples of mice by flowcytometry method, using the FITC anti-mouse CD4 antibody marker.

ELISA test

Measurement of IgG levels from mice serum samples using the ELISA method using an ELISA kit (Mouse IgG, Bioassay Tech, Lab).

Data analysis

Statistical analysis using Kruskal Wallis and Mann Whitney test with a confidence level of 0.05 ($p<0.05$).

RESULTS

Identification and selection In-Silico Epitope-Based SARS-CoV-2 Virus (SARS-CoV-2 spike glycoprotein, SARS-CoV-2 envelope, and SARS-CoV-2 membrane)

This protein is known to have an important role in host receptor recognition, viral entry, and pathogenicity. Proteins with antigenic scores greater than 0.8 (Table 1) were further used for epitope prediction for vaccine design.

Molecular Docking Results

The results of the molecular docking test can be seen from the value of the binding affinity. This molecular docking method uses a ligand in the form of an epitope and a receptor in the form of a Human Leukocyte Antigen (HLA). There are several HLAs used in this study, including HLA-B*44:03 (GDP ID: 3KPN) and HLA DRB-1*04:01 (GDP ID: 6BIJ). The molecular docking results for HLA-B*44:03 (GDP ID: 3KPN) with S, E, and M epitopes are shown in the following table:

The molecular docking results for HLA DRB-1*04:01 (GDP ID: 6BIJ) with S, E, and M epitopes are shown in the following table:

ISCOM Analysis Results using TEM microscope

ISCOM examination using a TEM microscope was performed to show that the epitope was conjugated with ISCOM (marked by a white sphere). The results of the examination are shown in Figure 2 that

80% of the peptides have been conjugated with ISCOM.

Examination Results Amount NK cells Using Flowcytometry

Figure 3 shows the number of NK cells as indicated by a pink image using a CD4+NK cell marker.

The results of the research were conducted to give an oral in-silico epitope-based SARS-CoV-2 effect on increasing the number of NK cells in mice providing descriptive value information from quantitative flow cytometry analysis using the Cell Quest Pro software. which is shown in Table 5, where the NK cell values obtained the highest average in P3 while the lowest average in the control was almost not much different from P4.

Results of Examination of Serum IgG Levels using ELISA

The results of research conducted to give an oral in silico epitope-based SARS-CoV-2 effect on increasing serum IgG levels in mice, providing descriptive value information as follows:

Based on Figure 5, it was found that the serum IgG values increased in the P1, P2, and P3 groups with the highest mean in P5 and the lowest in the control group.

DISCUSSION

The in-silico mapping method on the *SARS-CoV-2* virus has also been carried out by other researchers such as in Kalita et.al (2020), and Joshi et.al. (2021), and succeeded in finding the epitope of the SARS-CoV-2 virus, but none has been followed up with experimental animals.[2,8]

Our in-silico epitope-based *SARS-CoV-2* virus successfully carried out molecular docking and molecular dynamics. The results of protein sequence selection show that our epitope sequences have gone through the alignment process via BLAST on NCBI. Our research found that the most influential HLA according to the results of the IEDB-AR was HLA I represented by HLA-B*44:03 (GDP ID: 3KPN), and HLA II represented by HLA DRB-1*04:01 (GDP ID: 6BIJ). The results as seen in Table 2 of the molecular docking test between HLA I and the Spike, Envelope, and Membrane epitope showed that epitope B (-8.9 kcal/mol) had the lowest bond energy among the other two epitopes (S epitope and combined epitope S, E, and M). Molecular docking is one of the methods in in-silico testing that can predict the interaction between ligands and receptors. The more stable the ligand-protein interaction is reflected by the lower the score (minus).[9]

According to the results of our research exploration, it was found that the in-silico epitope selected in Table 3 is known to be immunodominant, non-toxic, and non-allergenic.[2] We named these three epitopes the S, E, and M epitopes to determine the immune response when given orally in mice by looking at the cellular immune response from the measurement of NK cells in the spleen and the humoral immune response of serum IgG in mice. Due to the oral administration of the epitope, it requires adjuvants and ISCOM is the choice.

The successful binding between the epitope and ISCOM that we selected is shown in Figure 1 from the results of the examination using TEM. The results of the TEM examination show the ISCOM image as an empty ball, but if this ball has contents (white color) it shows that ISCOM has bound or succeeded in conjugating with the epitope. If the number of balls that contain this is more than 80%, the

Table 1. Exploration Results *in silico epitope-based SARS-CoV-2 virus*

No	Sequence Epitope	Long	Information
1	FLVLLPLVSSQCVNL	15	*S epitope (Spike)*
2	VNSVLLFLAFVVFLLVTLASS	21	*E epitope (Envelope)*
3	LYIIKLIFLWLLWPVTLACFVLAAVY	26	*M epitope (Membrane)*

Note: F: Phenylalanine; L: Leucine; V: Valine; P: Proline; S: Serine; Q: Glutamine; C: Cysteine; N: Aspragine; A: Alanine; T: Threonine; I: Isoleucine; Y: Tyrosine

Table 2. Analysis Results Bond Epitope and HLA I: HLA-B*44:03 (GDP ID: 3KPN)

Epitope	Binding Affinity Score (kcal/mol)	RMSD ub
S	-8.7	2004
E	-8.9	17.764
M	-5.8	10.50

Notes: FLVLLPLVSSQCVNL(S) epitope, VNSVLLFLAFVVFLLVTLASS (E) epitope, and LYIIKLIFLWLLWPVTLACFVLAAVY(M) epitope.

Table 3. Analysis Results Bond Epitope and HLA II: HLA DRB-1*04:01

Epitope	Binding Affinity Score (kcal/mol)	RMSD ub
S	-7.4	11.678
E	-9.6	2.184
M	-6.5	13.40

Notes: FLVLLPLVSSQCVNL(S) epitope, VNSVLLFLAFVVFLLVTLASS (E) epitope, and LYIIKLIFLWLLWPVTLACFVLAAVY(M) epitope.

Table 4. Analysis Results Antigenicity, Toxicity, and Allergenicity Test the epitopes

Epitope	Epitope Sequence	SVM	TP	AlP	AnP	HP	WM
Spike (S)	FLVLLPLVSSQCVNL	-1.01	NT	NA	1.0416	-1.07	1645.27
Envelope(E)	VNSVLLFLAFVVFLLVTLASS	-1.45	NT	NA	1.1202	-1.24	2253.09
Membrane(M)	LYIIKLIFLWLLWPVTLACFVLAAVY	-1.34	NT	NA	1.0532	-1.49	3083.33

Notes: SVM (Support Vector Machine), TP (Toxin Prediction), NT (Non-Toxic), AlP (Allergenicity Prediction), NA (Non Allergic), AnP (Antigeniy prediction), HP (Hydrophilicity), MW (Weight Molecule), F (Phenylalanine), L (Leucine), V (Valine), P (Proline), S(Serine), Q (Glutamine), C (Cysteine), N (Asparagine), A (Alanine), T (Threonine), I (Isoleucine), and Y (Tyrosine).

Analysis Results Antigenicity, Toxicity, and Allergenicity Test

Epitope Visualization Results

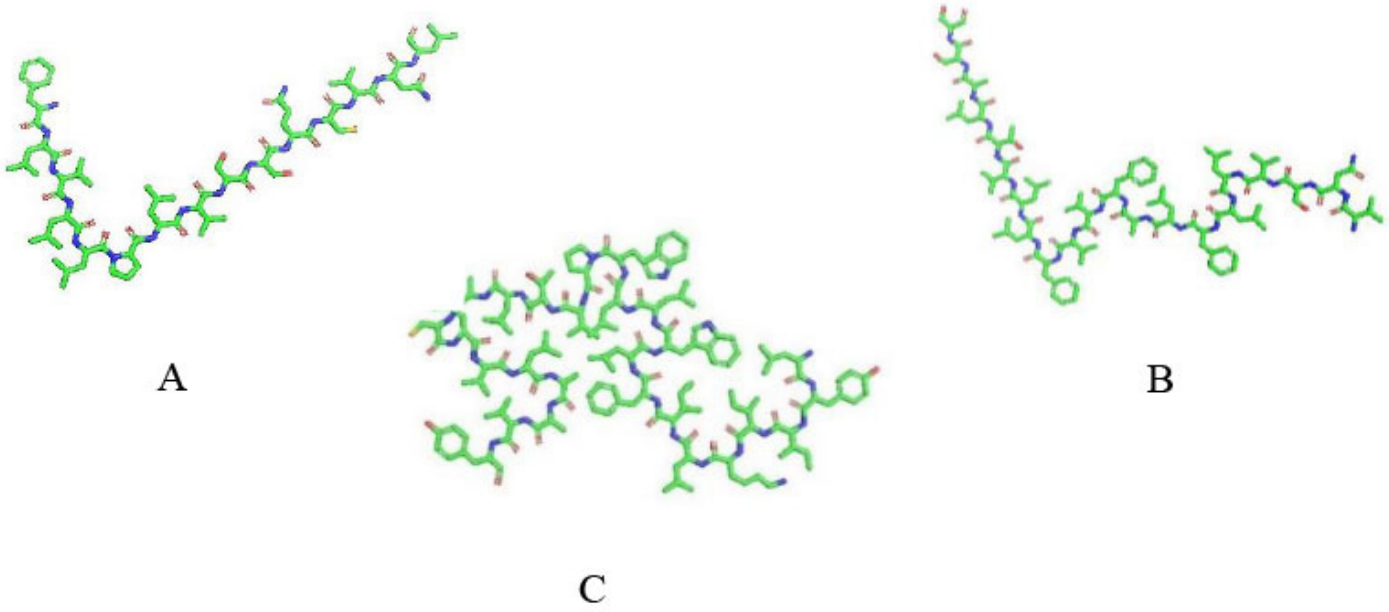

Figure 1. Visualization results design in-silico epitope-based SARS-CoV-2 virus. The structure epitope was designed using ChemSketch and visualized with PyMOL software.
A. S epitope (FLVLLPLVSSQCVNL), **B.** E epitope (VNSVLLFLAFVVFLLVTLASS), and **C.** M epitope (LYIIKLIFLWLLWPVTLACFVLAAVY)

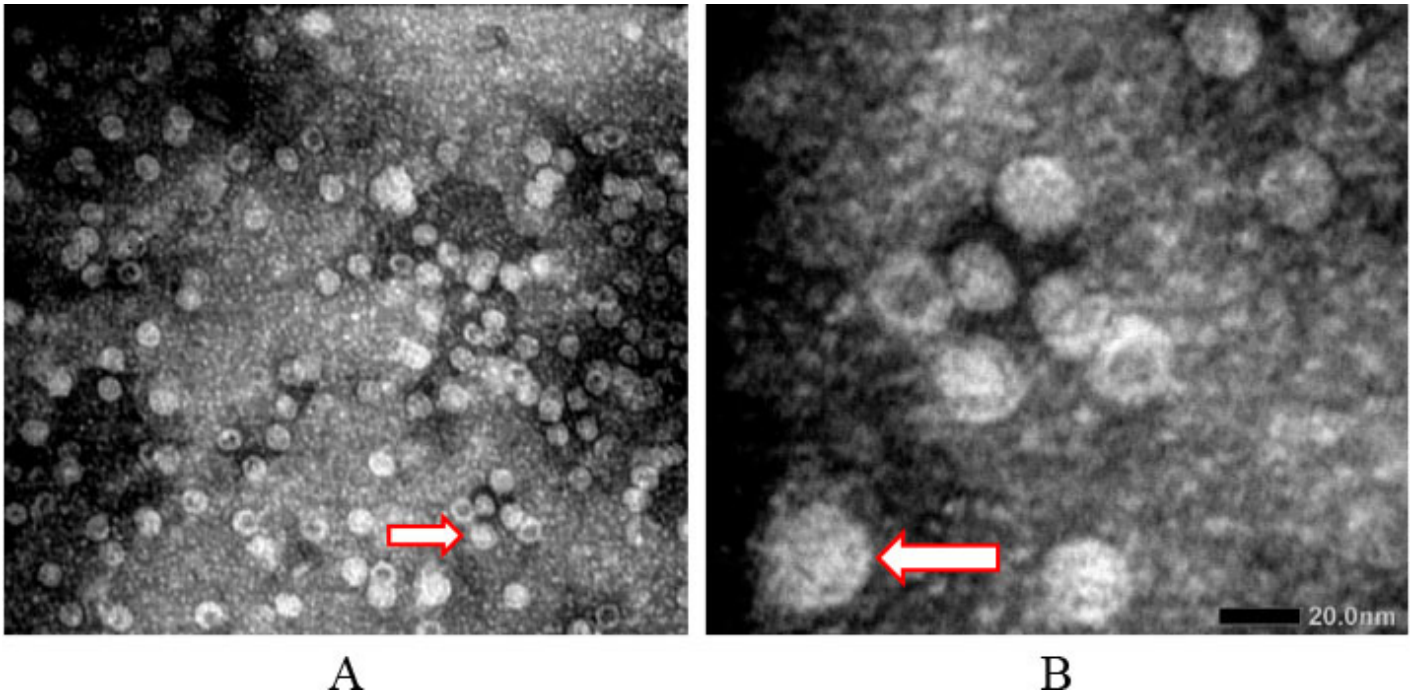

Figure 2. Results of ISCOM examination with a TEM microscope.
Notes: (A) Epitope conjugation and ISCOM 26nm from a 100 nm scale (40,000 times magnification) (B) Epitope and ISCOM conjugation 33nm from a 20nm scale (150,000 times magnification).

research is feasible to continue.[10]

Cellular adaptive immune responses responsible for lysing microbes/SARS-CoV-2 include macrophages, NK cells, and CTLs. The highest NK cell values were observed in the group given the S epitope. The results of this study are the same as the previous study, namely a study with Balb/c mice infected with SARS-1, which showed an increase in NK cells, immature macrophages, and dendritic cells in the lungs. mouse lung.[11] The spike protein plays a role in increasing the average number of NK cells because the spike protein (S) is responsible for receptor binding and viral membrane fusion, but also acts as the main antigen for humoral and cellular immunity.[12]

A previous study showed that NK cells can kill microbes.[13] In this response, the IgG value was lower in the group that was given the S epitope compared to the group that was given the SEM epitope. This can be explained in the study of Dai and Gao (2021) who said several T cell epitopes have been identified in M and E proteins in studies on immunity to *SARS-CoV* and *MERS-CoV*. It said the M and E proteins could help expand T-cell responses and increase cross-protection if they were included in the SARS-CoV-2 vaccine.[14]

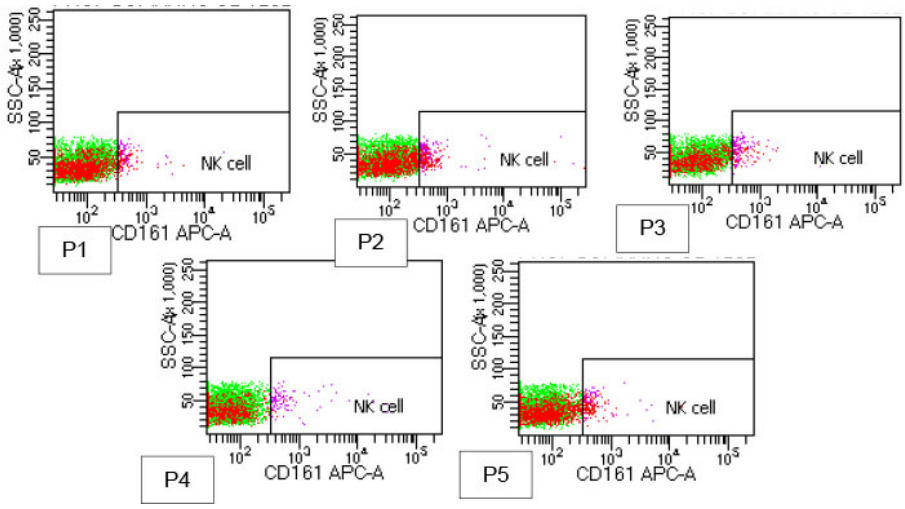

Figure 3. Results of flowcytometry analysis of NK cell levels using CD4+ marker. **Notes:** Treatment without given epitope (P1), ISCOMS (P2) epitope S (P3), epitope EM (P4), and epitope SEM (P5), Green color: Lymphocyte Cells; Purple color: NK cells; Red color: CD4+.

Table 5. Average number of NK cells per 0.07 g spleen of Balb/C mice after oral exposure to in-silico epitope-based SARS-CoV-2 virus

Treatment	Mean ± SD	Sig.
P1	1.16 ± 0.05	a
P2	1.24 ± 0.33	a
P3	4.08±0.16	c
P4	1.18±0.11	a
P5	2.16±0.17	b

Notes: Treatment without given epitope (P1), ISCOMS (P2) epitope S (P3), epitope EM (P4), and epitope SEM (P5). a,b, and c show different p-values

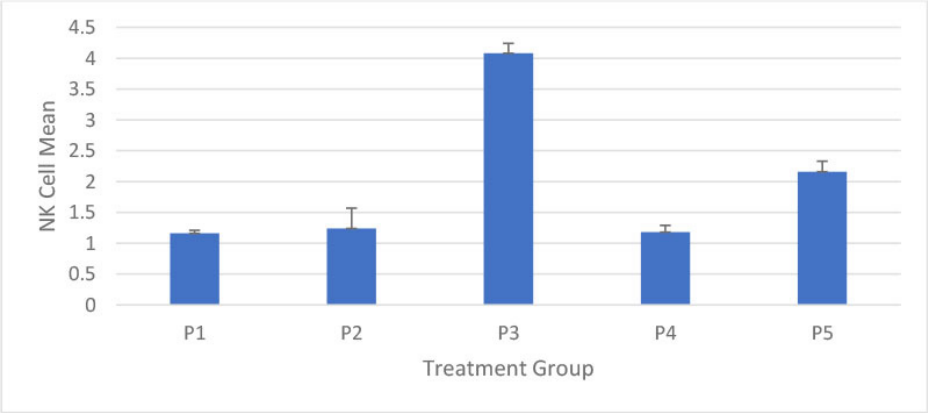

Figure 4. Flowcytometry Level Results of NK cells per 0.07 g spleen of Balb/C mice after oral exposure to in-silico epitope-based SARS-CoV-2 virus **Note:** Treatment without given epitope (P1), ISCOMS (P2) epitope S (P3), epitope EM (P4), and epitope SEM (P5).

CONCLUSION

In silico, SARS-CoV-2-based epitope found 3 epitopes of SARS-CoV-2 virus derived from spike protein FLVLLPLVSSQCVNL(S) epitope, envelope VNSVLLFLAFVVFLLVTLASS (E) epitope, and membrane LYIIKLIFLW-LLWPVTLACFVLAAVY (M). If three epitopes using the ISCOM adjuvant when administered orally, it was found to increase cellular immune response with NK cell markers and humoral immunity with serum IgG markers in mice.

CONFLICT OF INTEREST DECLARATION

The authors declare there is no conflict of interest associated with this study.

FUNDING

None.

ETHICAL STATEMENT

Committee Ethics Faculty Medicine Universitas Brawijaya Malang, Indonesia agrees to study this (Number Approval 044-KEP-UB-2020).

AUTHOR CONTRIBUTION

All authors contributed equally to this study.

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Table 6. Serum IgG levels in ng/ml, Balb/C mice after oral exposure to in silico epitope-based SARS-CoV-2 virus

Treatment	Mean ± SD	Sig.
P1	2.728 ± 0.313	a
P2	3.826 ± 0.206	b
P3	3.448 ± 0.462	b
P4	4.998 ± 0.409	c
P5	5.260±0.159	c

Note: Treatment without being given an epitope (P1), ISCOMS (P2), an S epitope (P3), an EM epitope (P4), and an SEM epitope (P5). a,b,c show different p-value.

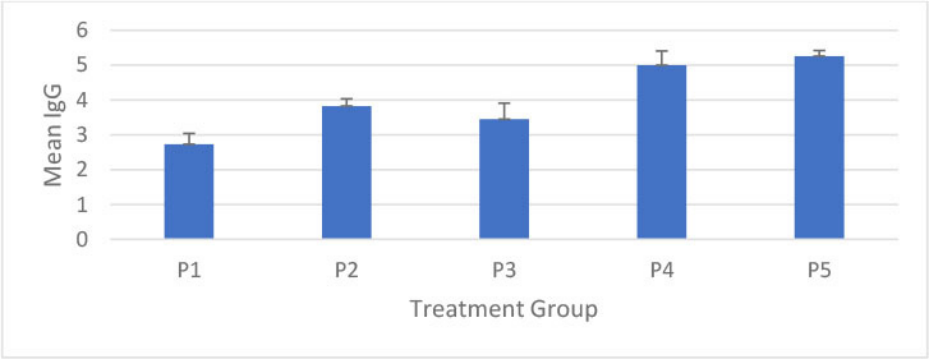

Figure 5. Serum IgG Levels in Mice Balb /c post Exposure in-silico epitope of SARS-CoV-2 virus orally. **Note:** Treatment without given epitope (P1), ISCOMS (P2) epitope S (P3), epitope EM (P4), and epitope SEM (P5).

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