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New potent epitopes from *Leptospira borgpetersenii* for the stimulation of humoral and cell-mediated immune responses: Experimental and theoretical studies

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A B S T R A C T

The rhKU_Sej_LRR_2271 protein is introduced as one of the leptospiral vaccine candidates since it contains the predicted immunogenic epitopes. The *in-silico* sequence and structural based analysis have been used in this study to analyze MHC class I and class II-restricted epitopes of the protein. Six epitope prediction programs were employed, which the epitopes with high prediction scores from each program were aligned. The 21 of potentially unrestrained epitopes with prediction scores above the cut-off value from at least two prediction programs were selected. The 3D-molecular modeling, docking, and molecular dynamic simulation were performed to evaluate the affinity binding between peptide-MHC complex and T-cell receptor. One promising epitope, which is 171-LLFLPLIKI, showed the potency in binding to both MHC class I and II alleles. Two newly designed peptides containing epitopes which can bind to over 3 of MHC alleles, LL17:171-LLFLPLIKILYVDRNKL-187 and SL19:209-SLNSGIKALPFNYEKLVNL-227, significantly increased interferon-gamma (IFNg)-producing specific T-cell responses in the rhKU_Sej_LRR_2271 immunized rabbits compared to nonimmunized rabbits. The LL17 peptide can also induce interferon-gamma-producing specific $CD4^+$ T-cell responses in the immunized rabbits. For the evaluation of humoral immune responses, the immunized rabbits have a significantly greater amount of specific IgG in plasma than the nonimmunized rabbits. *Ex-vivo* study of T-cell responses in animal model using flow cytometry confirmed an accomplishment of the theoretically *in-silico* analysis for discovering potential T-cell epitopes of the protein. The results show that the rhKU_Sej_LRR_2271 protein containing promiscuous T-cell epitopes, which can induce both humoral and cell-mediated immune responses, is a prospective protein candidate for leptospiral vaccine development.

1. Introduction

Leptospirosis is a bacterial septicemic infectious disease caused by pathogenic leptospires which can cause diseases in humans and animals especially livestock in all parts of the world. The disease is a ubiquitous zoonosis which is associated with chronic infected carrier animals. Leptospira can be transmitted by direct contact with the infected animals and can be transmitted by indirect contact with contamination of water and soil by urine from the infected animals. Pathogenic *Leptospira* species has been identified into over 260 immunologically distinct serovars [1]. Within these pathogenic species, *Leptospira borgpetersenii* serovars Sejroe is an important serovar causing cattle infection in Thailand [2]. Researchers have focused on the vaccine development for preventing the leptospiral infections for animal using bioinformatics tools and an experimental study in animal model.

In order to develop the effective vaccine, the mechanisms of the

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protective immunity against *leptospires* in both cell-mediated and humoral immune responses have to be clearly revealed. A killed vaccine was developed using *L. borgpetersenii* serovar Hardjo for cattle. It can stimulate a significant level of interferon-gamma (IFNg)-producing cells and significant numbers of IFNg-producing $CD4^+$ T-cells following vaccination. These results show the significant finding of inducing a sustained T helper type 1-mediated immune response after vaccination [3].

This study aims to identify the immunogenic epitopes that can stimulate the protective immune responses against leptospirosis by using computational bioinformatics, immunogenic prediction programs, and the *ex vivo* experiment in animal models. We have identified and successfully expressed a recombinant hybrid KU_Sej_LRR_2271 (rhKU_Sej_LRR_2271) LRR protein [4] from KU_Sej_R21_2271 gene (accession number JX522460) of *L. borgpetersenii* serovars Sejroe. We hypothesized that *L. borgpetersenii* rhKU_Sej_LRR_2271 hybrid protein could be the potential targets to induce the effective immune response against leptospirosis by generating strong humoral immune response and cell-mediated immune responses including the functional $CD4^+$ and $CD8^+$ T-cell responses.

To examine the hypothesis, we performed the *in-silico* analysis to predict T-cell epitopes on the rhKU_Sej_LRR_2271 protein based on binding affinity of peptide to Major Histocompatibility Complex (MHC) class I and II molecules, and the peptide-MHC (pMHC) recognition by T cell. T-cell epitopes were identified by six online programs and the binding scores for each epitope from those programs were all aligned. The unrestrained epitopes with prediction scores above the cut-off value from at least two software were selected. In order to evaluate the recognition of pMHC complex by T cell receptor (TCR), the computational analysis [5,6] with 3D modeling, molecular docking (MD) [7,8], and molecular dynamic simulation (MDS) [9] were used. The prediction results revealed the potentially promiscuous epitopes that can bind to the common HLA alleles in the database.

For the measurement of T-cell responses, peptides containing epitopes predicted by MD and MDS evaluations were designed and synthesized. These peptides were selected based on the criteria of MHC binding with at least three MHC alleles. *Ex vivo* T-cell responses were evaluated from rabbits immunized with rhKU_Sej_LRR_2271 protein by measurement of intracellular interferon-gamma (IFN-gamma)-producing T cells using flow cytometry. Additionally, the humoral immune responses were evaluated by the measurement of specific IgG in plasma from the immunized rabbits.

The results strongly demonstrated that the bioinformatic tools of computer-based *in-silico* study and structural based analysis can be used to discovery the potentially promiscuous T-cell epitopes that can induce the strong immune responses. It concludes that the predicted epitopes derived from rhKU_Sej_LRR_2271 protein can effectively stimulate both humoral and cell-mediated immune responses in the immunized rabbits which can be used for the development of effective vaccine. This study showed the advantage of using both computational analysis and the *ex vivo* experiment in animal models for the development of leptospiral vaccine.

2. Methods

2.1. Prediction of T-cell epitopes by in-silico analysis

The T-cell epitopes were identified from the rhKU_Sej_LRR_2271 leucine rich repeat protein by MHC binding prediction programs. The amino acid sequence was obtained from NCBI databases (GenBank accession number JX522460). The prediction of epitopes binding to MHC class II molecules was done using motif matrices based SYFPEITHI, the additional method MHCPred [10], artificial neuron networks based NetMHCII 2.2 and quantitative matrices based Propred [11]. For the prediction of epitopes binding to MHC class I molecules, the protein sequence was subjected to matrices based Propred I [12], artificial neuron networks based NetMHC 3.4 [13], SYFPEITHI and MHCPred.

According to the most frequent MHC alleles identified in database [14,15], the epitopes were subsequently evaluated the binding affinity to MHC class II allele including HLA-DRB1*0101, HLA-DRB1*0301, HLA-DRB1*0401, HLA-DRB1*0701, HLA-DRB1*1101, and HLA-DRB1*1501, and to MHC class I allele including HLA-A*0201, respectively.

The accepted criteria for selection of the candidate epitopes are as follows: 1) Ranking score above the threshold based on precedent literatures and program instructions defining the score >25 for SYFPEITHI [16], threshold > 3% for Propred. Binding affinity less than 50 nM was indicated as the strong binding affinity and 50–500 nM was indicated as the weak binding affinity for NetMHCII 2.2, NetMHC 3.4 and MHCPred. 2) The epitopes that pass the criteria 1) with at least two methods will be chosen for further investigation. To confirm the presence of these epitopes, the previously identified epitopes from leptospiral known antigenic proteins including LipL32, LigA and OmpL1 were used to perform the sequence-based epitope prediction [17–22].

2.2. Computational docking and simulation of the predicted T cell epitopes on MHC molecules and T cell receptors recognition

2.2.1. Preparation of peptide epitopes based on MHC structures

The 3D structures of the predicted epitopes were built from the homology model of LBJ_2271 and whole protein structures of rhKU_Sej_LRR_2271 published in the previous study [4,23]. The peptide sequences were aligned and the short peptide structures were retrieved from the protein structure modeling. The epitopes' structures of rhKU_Sej_LRR_2271 protein were built by Modeler 9v8 in Discovery Studio (DS) 3.0 using a homology model of the LBJ_2271 protein as the template structure. The conformations of peptide side chain were optimized in side chain refinement protocol DS 3.0. The PDB structures of MHC molecules were obtained from the Protein Databank (PDB) which excluded the original bound peptides. The PDB identification numbers of MHC molecules including DRB*0101, DRB*0301, DRB*0401, DRB*1501 and A*0201 alleles were 1SJE, 1A6A, 2SEB, 1BX2 and 1AO7, respectively. The structure of DRB*0701 and DRB*1101 alleles were not available in the database. Therefore, the tertiary structures were built by homology modeling. Amino acid sequence of DRB*0701 and DRB*1101 alleles were obtained from the Immune Polymorphism Database (IPD). The sequences were subjected to Swiss model template identification tools and aligned with amino acid sequence of template structure. The homology modeling was performed in DS 3.0 and the reliability of model structures was validated in Swiss model structure assessment.

2.2.2. Molecular docking of peptides on MHC class I and II molecules and docking of T cell receptor (TCR) on a peptide-MHC complex

Molecular docking was performed to investigate the interaction of specific peptide with MHC class either I or II molecules and docking of TCR on peptide-MHC complex (pMHC). The predicted epitopes were docked with MHC class I and II molecules followed by docking of the TCR molecule on pMHC complex using dock proteins protocols in DS 3.0 Client. ZDOCK is a grid-based global search algorithm used as a Fast Fourier Transform technique and a novel shape complementarity scoring function. RDOCK is an algorithm based on the CHARMM forcefield energy minimization scheme for removing van der Waals clashes and optimizing interactions of polar and charge. The scoring system is comprised of a CHARMM electrostatic energy term and a desolvation energy term [7,8]. Firstly, we docked a peptide into a MHC molecule by ZDOCK. The distance restraint for interfacial residues was kept at 10 Å and the angular step size for rotational sampling of ligand orientations was 15 Å. The conformation was considered similarity to the original PDB structure. The orientation of the docked peptide on the MHC binding groove should be similar as the native structure. Then, the chosen dock poses were further refined using the RDOCK protocol. The poses with the lowest RDOCK score and the highest ZDOCK score of each

pMHC complex were kept for further docking with a TCR. Docking of a pMHC complex on a TCR was likewise performed as in pMHC docking. Two PDB molecules were retrieved from the PDB database for T cell receptors of the $CD8^+$ T cell (PDB ID: 1AO7) and the $CD4^+$ T cell (PDB ID: 2IAM). The conformation of docking complex should be similar to the native structure in which peptide interacts with both TCR alpha and beta chains. The ZDOCK score, RDOCK score and H-bond between receptor and ligand were determined for comparative analysis of the docking complex.

2.2.3. Molecular dynamic simulation

Molecular dynamics (MD) simulations are the standard tools for investigating biomolecular interactions dynamically. Simulations aid an understanding of biochemical processes and give a dynamic dimension to structural data [9]. The characteristics of TCR/pMHC complexes were analyzed by simulation studies. Simulation was performed using CHARMM27 forcefield in GROMACs version 2021.1 program [24]. The docking complex formation of peptide-MHC complex and TCR were used as initial structures. The system was solvated in cubic box with TIP3P water model. To neutralize the system, chlorine and sodium ions were added into the system. Energy minimization was performed using the steepest descent minimization algorithm for 50000 steps with step size of 0.01 ns to remove bad contacts and to relax the system. The equilibration steps of the system were performed with NVT and NPT ensemble, respectively. For the NVT and NPT equilibration steps, the structure was equilibrated at 300 K for 100 ps with step size of 2 fs. All MD simulations were performed at 300 K and 1 bar for 50 ns with step size of 2 fs.

2.3. Animals

New Zealand White rabbits were purchased from the National Laboratory Animal Center, Mahidol University, Thailand. They were maintained at the Faculty of Veterinary Medicine, Kasetsart University, Thailand. All rabbits used in this study were female and age-matched at 10–12 weeks old. Three rabbits for each group were used as the experiment group (immunization) and the negative control group. Each rabbit was kept individually in a standard cage with ad libitum access to a commercial pelleted ration and drinking water. The animal experiment protocol was approved by Kasetsart University Institutional Animal Care and Use Committee (Kasetsart University-IACUC).

2.4. Cloning, expression and purification the recombinant KU_Sej_LRR_2271 protein

The rhKU_Sej_LRR_2271 protein was produced from pET160_hKU_R21_2271 plasmid in *Escherichia coli* BL21 Star™ (DE3) expression system as previously described [4]. Briefly, the protein expression was induced from pET160_hKU_R21_2271-transformed *E. coli* BL21 Star™ (DE3) by adding isopropyl β-D-1-thiogalactopyranoside (IPTG). The cell pellet was collected and disrupted by sonication. Total cell lysate was centrifuged at 12,000×*g* at 4 °C for 20 min. The insoluble proteins were collected and washed twice with LEW buffer and then resuspended in denaturing solubilization buffer (DS: 50 mM NaH_2PO_4, 300 mM NaCl, 8 M urea, pH 8.8) containing protease inhibitors (SigmaFast™ protease inhibitor cocktail tablet, Sigma-Aldrich™). The dissolved and solubilized proteins were purified by the immobilized metal ion affinity chromatography (IMAC) technique with Protino® Ni-TED Resin (Macherey-Nagel, Germany). The purified proteins were saved at −20 °C until use.

2.5. Immunization protocol

Age-matched New Zealand White rabbits ($n = 3$) were injected subcutaneously with the mixture of 500 μg rhKU_Sej_LRR_2271 recombinant protein and complete Freund adjuvant for the first immunization. For the second and the third immunizations, they were administered intramuscularly and subcutaneously with the mixture of recombinant protein and incomplete Freund adjuvant, respectively. The second immunization was 14 days after the first immunization and the third immunization was 28 days after the second immunization. As the negative control group, three rabbits were immunized with mixture of phosphate buffer saline and adjuvants on the same schedule as the vaccine course. The whole blood samples were collected to isolate peripheral blood mononuclear cells (PBMC) and plasma at day 0, 14, 28 and 56 after the first immunization. Freshly prepared PBMC was used to evaluate the specific T cells functions using flow cytometry. The plasma was collected and kept at −80 °C until use.

2.6. Peripheral blood mononuclear cells (PBMCs) isolation

Whole blood (8 ml) was drawn from a central artery into EDTA tubes. Whole blood was collected at four time-points to evaluate T-cell responses consisting of before the first immunization as baseline, 14 days after the first immunization, 14 days after the second immunization, and 14 days after the third immunization. PBMCs were isolated by gradient centrifugation using Ficoll-Hypaque reagent (Amersham). The isolated PBMCs were washed with phosphate buffer saline (PBS) and then resuspended in R10 medium (RPMI1640 supplemented with 10% fetal bovine serum; FBS, penicillin/streptomycin, and HEPES) at the concentration of 10^6 cells/ml [25,26] for the measurement of specific T-cell responses.

2.7. Measurement of specific T-cell responses by flow cytometry

Freshly prepared PBMCs were used to evaluate the function of T-cell responses by measurement of intracellular IFNg production using flow cytometry [25,26]. Briefly, for the IFNg-producing T cells measurement, 10^6 PBMCs were stimulated with an individual peptide at a concentration of 10 μg/ml in R10 medium containing protein transport inhibitor (Monensin, Becton Dickinson) for 6 h at 37 °C, 5%CO_2. All peptides were synthesized and HPLC purified to the purity of 90%–95% (Mimotope, Australia). Phytohemagglutinin (PHA, Sigma-Aldrich) and R10 medium were used as positive and negative controls, respectively. For the cell surface staining, cells were stained with APC-conjugated mouse anti-rabbit CD4 (clone KEN-4, Abcam) and FITC-conjugated mouse anti-rabbit CD8 (clone 12. C7, Serotec) in PBS containing 1% FBS and 0.1% sodium azide (FACS buffer) for 45 min at 4 °C. Subsequently, cells were permeabilized using Cytofix/Cytoperm solution (Becton Dickinson) for 40 min at 4 °C. For intracellular staining, the permeabilized cells were stained with PE-conjugated mouse anti-bovine IFNg (clone CC302, Serotec) in Cytofix/Cytoperm wash buffer for 30 min at 37 °C. The stained cells were finally fixed in 1% Paraformaldehyde (Sigma-Aldrich) solution and at least 500,000 cells were acquired by flow cytometry (CytoFlex, Beckman Coulter). All antibodies in this study were used in the optimal concentration after the antibody titration and compensation control was properly performed. For the measurement of IFNg production, the isotype control was used as the control. The specific T-cell responses stimulated with peptide were subtracted from the negative control and only T-cell responses above its background level were considered as the positive values.

2.8. Evaluation of specific humoral immune responses by using ELISA assay

Humoral specific IgG against rhKU_Sej_LRR_2271 protein in the immunized and control rabbit groups were evaluated by using an Enzyme-Linked ImmunoSorbent Assay (ELISA). In brief, the rhKU_Sej_LRR_2271 protein (7.5 μg/ml) in carbonate-bicarbonate buffer (CBB) was coated on 96-well plates (100 μl/well), incubated at room temperature for 2 h and then washed three time with 1X PBS supplemented with tween (PBST). The 300 μl of 2% BSA in PBST was added

and incubated at room temperature for 1 h. Then, 100 μl of 1:1600 dilution of rabbit plasma or PBS was added and incubated at 37 °C for 1 h. After that, the 2.7% Hydrogen Peroxide was added and incubated for 5 min. After a washing step, the goat anti-rabbit IgG–HRP diluted to 1:20000 (Santa Cruz Biotechnology) was added to each well and incubated at 37 °C for 1 h. The 100 μl of 1-Step™ Ultra TMB-ELISA substrate solution (Thermo Scientific) was subsequently added and incubated for 5 min in the dark. Then, 2 M sulfuric acid was added to each well. Absorbance was measured by an ELISA reader (Biotek instruments Inc., USA) at 450 nm. Optical Densities (ODs) of the background were subtracted from samples' ODs and the results compared between the immunized and control rabbit groups.

2.9. Statistical analysis

The statistical analysis was performed using Student's t-test to compare the level of antibody production between control and immunized rabbits. One-way ANOVA (Krusskal-Wallis test) was used to compare the frequency of IFNg-producing T cells. Data were calculated as the median values. The *p* values of less than 0.05 were considered as statistically significant.

3. Results

3.1. Identification of T-cell epitopes

Sequence-based method was initially used for a screening of T-cell epitopes from the rhKU_Sej_LRR_2271 protein. Six online epitope prediction programs were utilized to predict T-cell epitopes. For the prediction of epitopes binding to MHC class II molecules, the sequence was analyzed against six HLA-DRB alleles. The results from NetMHCII 2.2, SYFPEITHI and MHCPred showed that the epitope binding with HLA-DRB*0101 allele had the highest binding affinity scores at 3.7, 36 and 0.78, respectively. The Number of epitope binding with this HLA allele was higher than other HLA-DRB alleles in NetMHCII2.2 and MHCPred (Table 1). As shown in Table 2, the highest prediction scores of epitopes for each allele from Propred program were compared with epitope prediction scores of known leptospiral antigenic proteins; LipL32, LigA and OmpL1. The rhKU_SEJ_LRR_2271 scores have approximate value with the known antigenic protein. Importantly, the Propred scores of predicted epitopes binding with HLA-A*0201 and HLA- DRB*1501 alleles were higher than LipL32 and LigA prediction score while equal to the highest score of OmpL1 (Table 2). For the evaluation of other potentially effective T-cell epitopes, the alignment of the prediction scores from each epitope with above the cut off value from each program were performed. The 21 epitopes having prediction score above the threshold from at least 2 prediction programs were selected and classified as the potent T-cell epitopes, as shown in Table 3 (3.1 and 3.2). Interestingly, the epitope at amino acid position 171-LLFLPLIKI was able to bind to both MHC class I (A*0201) and MHC Class II (DRB0*101, DRB*0301, DRB*0701, DRB*1101 and DRB*1501) alleles.

Table 1
The epitope prediction of rhKU_Sej_LRR_2271 protein by bioinformatic programs revealed the number of epitopes with the prediction score above cut off values.

Allele	SYFPEITHI		MHCPred		NetMHC		Propred	
	A	b	a	b	a	b	a	b
DRB*0101	8	36	108	0.78	58	3.70	11	1.70
DRB*0301	5	29	n	n	11	7.20	6	4.80
DRB*0401	10	28	1	32.28	3	34.30	10	3.80
DRB*0701	5	30	5	10.40	16	4.30	12	6.50
DRB*1101	0	25	n	n	38	6.60	12	4.40
DRB*1501	6	34	n	n	7	42.70	12	5.70
A*0201	6	29	6	16.98	4	19.00	2	6.10

a = Number of the predicted epitopes; b = The highest prediction score.
n = This allele is not included in the prediction program.

Table 2
Comparisons the highest Propred prediction scores between the epitopes from rhKU_Sej_LRR_2271 protein and epitopes from the known antigenic *Leptospira* proteins for each MHC allele.

MHC alleles	The highest Propred prediction scores			
	hKU_Sej_LRR_2271	LipL32	LigA	OMPL1
A*0201	6.1	5.5	4.3	6.1
DRB*0101	1.7	3.3	2.6	1.1
DRB*0301	4.8	4.4	5.2	4.8
DRB*0401	3.8	3.9	5.0	3.8
DRB*0701	6.5	5.5	7.0	6.5
DRB*1101	4.4	4.5	3.0	4.4
DRB*1501	5.7	5.5	5.5	5.7

3.2. Computational docking and simulation of the predicted T-cell epitope with MHC molecules and TCR recognition on pMHC complex

For the structural analysis of MHC binding peptide, 3D structure of 21 of the predicted T-cell epitopes were generated based on homology model of LBJ_2271 and rhKU_Sej_LRR_2271 hybrid protein [4,23]. Amino acid sequences of HLA-DRB*0701 and DRB*1101 alleles were obtained from IMGT/HLA with accession number HLA00719 and HLA00751, respectively. The template used for HLA-DRB*0701 allele is the PDB id: 1SEB, the complex of human MHC class II glycoprotein HLA-DR1 and the bacterial super-antigen, with 89.0% sequence identity while the template for HLA-DRB*1101 allele is the PDB id: 1A6A, the structure of an intermediate in MHC class II maturation: CLIP bound to HLA-DR3, with 94.2% sequence identity.

After the prediction of MHC classes I and class II binding epitopes by sequence analysis, the structural based analysis was used to confirm the prediction of the 21 of selected peptides as T-cell epitopes. The selected dock possessed similar structural orientation with the original PDB structure of MHC molecule. N-terminus of peptide in binding groove was pointed toward C-terminus of alpha domain in MHC class I molecule and alpha-beta domain in MHC class II molecule. The pMHC complex with the best score was further docked with TCR. Docking score of the 7 pMHC/TCR complexes with high score and numbers of hydrogen bonds in each MHC allele are shown in Table 4. The "SLEELDLSL" peptide showed the highest docking score and number of hydrogen bond with MHC class I allele (A*0201). The "ILYVDRNKL" peptide exhibited the highest docking score and number of hydrogen bonds in complexed with MHC class II allele (DRB*0701). Structures of pMHC/TCR complexes which have the highest number of hydrogen bonds forming with the MHC class I and class II molecules were shown in Fig. 1. The "SLEELDLSL" peptide formed 15 hydrogen bonds with MHC class I molecule was demonstrated in Fig. 1A while the 10 hydrogen bonds were formed between the "ILYVDRNKL" peptide and MHC class II molecule, as shown in Fig. 1B. The residue involved in hydrogen bond interaction and distance were shown in Table 5. There are 2 hydrogen bonds at residue 1 and 3, and 3 hydrogen bonds at residue 8 on the "ILYVDRNKL" peptide. Only residue 1 of this peptide interacts with both MHC and TCR molecules. The "SLEELDLSL" peptide had 15 hydrogen bonds forming with MHC and TCR molecules. Residue 1 and 3 had the highest number of hydrogen bonds (4 bonds) among other residues. The residues 1, 2 and 4 interacted with MHC and TCR molecules while residue 3 of this peptide interacted only with the MHC molecule.

In order to investigate the flexibility of the complex structure and consistency of the predicted epitope, the MD simulation was performed on the complex of peptide/MHC/TCR. The root-mean-square deviation (RMSD), an indication of the stability of the complex structure, of the entire molecule during the production phase relative to the starting structures was reported. For the SLEELDLSL/MHC I (A*0201)/TCR complex, the low fluctuation of backbone atom in SLEELDLSL peptide complex with the RMSD value less than 2 Å was found after 35 ns as

Table 3
The predicted T-cell epitopes from the rhKU_Sej_LRR_2271 protein with the prediction score above threshold from at least 2 prediction programs were demonstrated. Epitope sequences and positions were reported with the prediction score 4 prediction programs for MHC class I (Table 3.1) and for MHC class II (Table 3.2) alleles.

Table 3.1/Table 3.2

Epitope position	Sequence	Table 3.1: Scores for MHC class I allele (A*0201) from each Prediction program[a]/ Table 3.2: Scores for MHC class II alleles (allele) from each Prediction program[a]			
		Propred I/Propred	MHCPred/MHCPred	NetMHC3.4/NetMHCII2.2	SYFPEITHI/SYFPEITHI

Table 3.1

Epitope position	Sequence	Scores for MHC class I allele (A*0201) from each Prediction program[a]			
		Propred I	MHCPred	NetMHC3.4	SYFPEITHI
94	KLSTVPEEV	4.78	–	19	–
110	KLDLRLNSL	4.23	–	–	28
166	ILPSELLFL	6.13	–	24	27
171	LLFLPLIKI	5.02	–	–	28
202	SLEELDLSL	–	–	72	27
209	SLNSGIKAL	3.90	–	–	29

Table 3.2

Epitope position	Sequence	Scores for MHC class II alleles (allele) from each Prediction program[a]			
		Propred	MHCPred	NetMHCII2.2	SYFPEITHI
53	FLVIGFVCF	5.5 (DRB*0701)	–	–	25 (DRB*0101)
55	VIGFVCFTA	0.68 (DRB*0101) 5.1 (DRB*1501)	–	–	30 (DRB*1501)
58	FVCFTASFD		0.78 (DRB*0101)	–	30 (DRB*1501)
134	LNLFGNDLT	4.0 (DRB*1501)	–	–	26 (DRB*0401) 28 (DRB*1501)
148	FSKLKNLKV	1.8 (DRB*0401)	3.5 (DRB*0101)	3.7 (DRB*0101)	29 (DRB*0101) 28 (DRB*0401)
151	LKNLKVLLA	0.7 (DRB*0101) 3.0 (DRB*0301) 3.0 (DRB*0401) 4.4 (DRB*1101) 3.7 (DRB*1501)	–	–	25 (DRB*0101)
154	LKVLLAGNN	–	10.4 (DRB*0701)	–	25 (DRB*0101)
164	FTILPSELL	1.7 (DRB*0101) 2.5 (DRB*0401) 5.6 (DRB*0701)	–	4.6 (DRB*0101)	36 (DRB*0101) 28 (DRB*0401) 26 (DRB*0701)
171	LLFLPLIKI	1.0 (DRB*0101) 3.0 (DRB*0301) 4.9 (DRB*0701) 2.6 (DRB*1101) 4.7 (DRB*1501)	–	–	26 (DRB*0101)
173	FLPLIKILY	3.5 (DRB*1101)	3.63 (DRB*0101)	–	26 (DRB*0301) 25 (DRB*1101)
179	ILYVDRNKL	5.4 (DRB*0701) 4.2 (DRB*1501)	2.34 (DRB*0101)	–	28 (DRB*0301) 26 (DRB*0401)
180	LYVDRNKLT	1.6 (DRB*0401)	–	–	27 (DRB*0301) 26 (DRB*0401)
181	YVDRNKLTL	1.9 (DRB*1101)	6.22 (DRB*0101)		
194	VEILASLSS	0.9 (DRB*0101) 3.8 (DRB*0401) 3.8 (DRB*1101)	–	–	33 (DRB*0101) 26 (DRB*0401) 24 (DRB*1501)
219	FNYEKLVNL	0.4 (DRB*0101) 4.3 (DRB*0701)	1.56 (DRB*0101)	–	–
227	LINLKRLNI	0.7 (DRB*0101) 3.4 (DRB*0301) 4.3 (DRB*0701) 3.9 (DRB*1101) 5.7 (DRB*1501)	3.92 (DRB*0101)	–	26 (DRB*0401)

[a] Binding scores reported in MHCPred and NetMHC are binding affinity (nM). The score reported in Propred is the log binding score, while SYFPEITHI is reported as peptide binding capacity score.

shown in Fig. 2A. From the plots, the average RMSD values of backbone TCR chains and SLEELDLSL peptide were about 2.58 and 2.88 Å, respectively, which are quite stable along the simulation times. In case of HLA-A*0201, the RMSD of all chain (alpha I, II, and III) revealed the similar tendency with the complex. However, the average RMSD value of the alpha I and II parts (residue 1–180) which are the parts bound with peptide and connected with TCR was quite low RMSD with the value of 2.30 Å indicating the stability of peptide binding along the simulation times. Whereas, the high fluctuation caused by the alpha III part (residue 181–274) which is reported as a part bound with β2 microglobulin. For the ILYVDRNKL/MHC II (DRB*0701)/TCR complex, the RMSD results from 50 ns simulations were shown in Fig. 2B. The

Table 4
Molecular docking of the epitope peptides binding to MHC molecules and T cell receptors. MHC alleles and peptides with the highest docking score and hydrogen bonds were shown.

Alleles	Epitope peptides	RDOCK score	Number of H-bonds
A*0201	SLEELDLSL	−21.980	15
DRB*0101	VEILASLSS	−13.226	9
DRB*0301	LLFLPLIKI	−14.965	7
DRB*0401	LNLFGNDLT	−13.934	6
DRB*0701	ILYVDRNKL	−17.200	10
DRB*1101	LKNLKVLLA	−9.996	8
DRB*1501	LINLKRLNI	−15.771	8

results revealed the stability of the complex along the simulations with the average RMSD of 2.15 Å. The separated chains also revealed the stability with the average RMSD values of 1.85, 1.94, 2.17, 2.17, and 1.72 Å for DRB*0701 alpha, DRB*0701 beta, ILYVDRNKL peptide, TCR alpha and TCR beta, respectively. The MD results indicated that SLEELDLSL/MHC I (A*0201) and ILYVDRNKL/MHC II (DRB*0701) complexes could bound strongly with the TCR. The snapshots at 0, 10, 20, 30, 40 and 50 ns of SLEELDLSL/MHCI/TCR and ILYVDRNKL/MHCII/TCR complexes along MD simulations were shown in Fig. 3A and B, respectively.

3.3. Designing peptides with the potentially promiscuous T-cell epitopes

T cell epitopes from rhKU_Sej_LRR_2271 protein which have the prediction score higher than the threshold value from at least 2 prediction programs (Tables 3.1 and 3.2) and have the highest molecular docking scores for the formation of pMHC/TCR complex (Table 4) were selected. These epitopes contain as promiscuous T-cell epitopes. Four new peptides including FN15, LL17, VL17, and SL19, were designed as shown in Table 6. These newly designed peptides contain overlapping epitopes that have high prediction score, and contain ability to bind various MHC alleles (Tables 3.1 and 3.2) with the highest docking scores (Table 4). All newly designed peptides are recommended as hypothetically unrestrained T-cell epitopes of the protein. As the 3D model structure of rhKU_Sej_LRR_2271 protein shown in Supplementary Fig. 1, the regions of the predicted epitopes which contain both MHC class I and class II-restricted epitopes were illustrated in red and yellow colors. The red color indicates LL17 peptide and yellow color indicates VL17 and SL19 peptides. The red region illustrates epitope at position 171–187 which is specific to MHC class I allele HLA-A*0201 and MHC class II alleles including HLA-DRB*0101, DRB*0301, DRB*0401, DRB*0701,

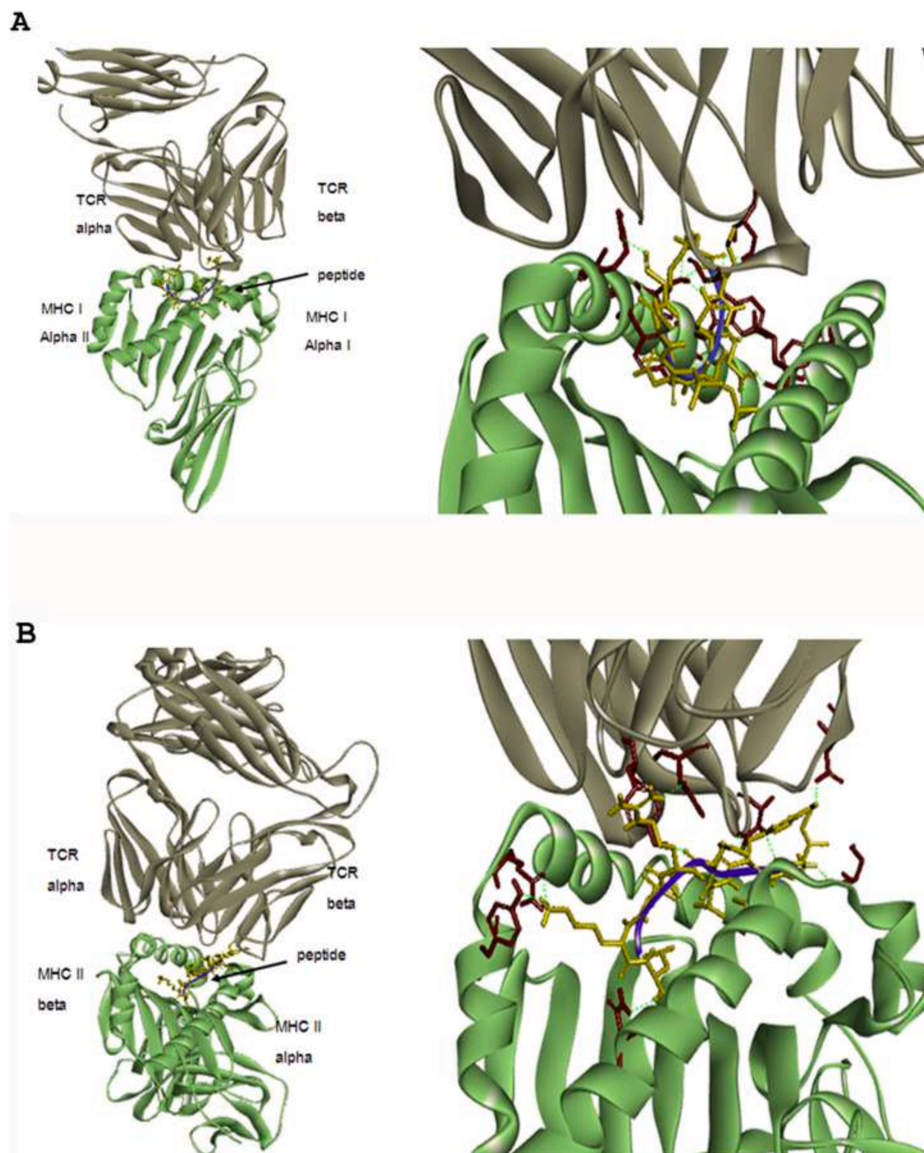

Fig. 1. Overall complex formation of pMHC/TCR. (**A**) Represents the complex formation of SLEELDLSL/MHCI/TCR. (**B**) Represents the complex formation of ILYVDRNKL/MHCII/TCR. TCR is shown in grey ribbon, MHC in green ribbon and peptide in blue ribbon and yellow stick. Amino acid residues of TCR and MHC involved in the formation of H-bond network were displayed in red stick. H-bonds was represented in green dashes. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 5
The interaction of pMHC/TCR complex in terms of hydrogen bonding.

Epitope peptide	MHC (alleles)	Residues involved in hydrogen bonding			Hydrogen bond distance (Å)
		Epitope	MHC allele	TCR	
SLEELDLSL	MHC class I: (A*0201)	SER1	THR163		1.95
			GLU166		2.10
			TRP167		1.87
				SER100	1.93
		LEU2	THR163 (HG1-O)		1.94
			THR163 (HN-OG1)		1.99
				GLY101	2.46
		GLU3	TYR59 (HH-OE1)		2.04
			TYR59 (HH-OE2)		2.27
			LYS66 (HZ1-O)		2.02
			LYS66 (HZ3-O)		2.20
		GLU4	TYR159		2.44
				GLY101	2.41
		ASP6	TYR99		2.29
		LEU9	GLN155		2.22
ILYVDRNKL	MHC class II: (DRB*0701)	ILE1		ALA93	2.38
			SER53		1.81
		TYR3		GLY92	2.14
				ASP56	2.36
		ASP5		TYR95	1.80
		ARG6		ASN30	2.31
		LYS8	TYR60		2.34
			GLN64		2.10
			ASN69		2.43
		LEU9	ASN69		2.30

DRB*1101 and DRB*1501. The yellow region illustrates epitope at position 194–227 which is specific to MHC class I allele HLA-A*0201 and MHC class II alleles including HLA-DRB*0101, DRB*0401, DRB*0701, DRB*1101 and DRB*1501. However, there is a predicted peptide, FN15, having high prediction score for only MHCII allele including HLA-DRB*0101, DRB*0301, DRB*0401, DRB*0701, DRB*1101, DRB*1501.

3.4. Assessment cell-mediated immune responses by stimulation of IFNg-producing T cells especially CD4$^+$ T cells in the rhKU_Sej_LRR_2271 immunized rabbits

To study the immunogenicity of the predicted T-cell epitopes on the rhKU_Sej_LRR_2271 protein, the measurement of specific T-cell responses was performed. Four newly designed peptides containing promiscuous T-cell epitopes indicated in Table 6 were evaluated the ability to stimulate *ex vivo* T cells by measurement of intracellular IFNg production. The frequency of IFNg-producing T cells including CD4$^+$ and CD8$^+$ T cells was measured before and after each immunization from New Zealand white rabbits. The results from all 4 predicted peptides were compared between the immunized group and control group (n = 3 each).

The stimulation of T-cell responses with two out of four predicted peptides which are LL17 and SL19 showed the significantly greater frequencies of *ex vivo* IFNg-producing T cells in the immunized rabbit group compared to the control group (p value $\leq$ 0.05) as shown in Fig. 4. In addition, one of these two responding peptides which is LL17 can induce the significantly greater number of IFNg-producing T cells in the immunized group than those of the control group after the second and third immunization as shown in Fig. 4A. Furthermore, the LL17-specific T-cell responses from the immunized group had greater frequencies of both IFNg-producing T cells and IFNg-producing CD4$^+$ T

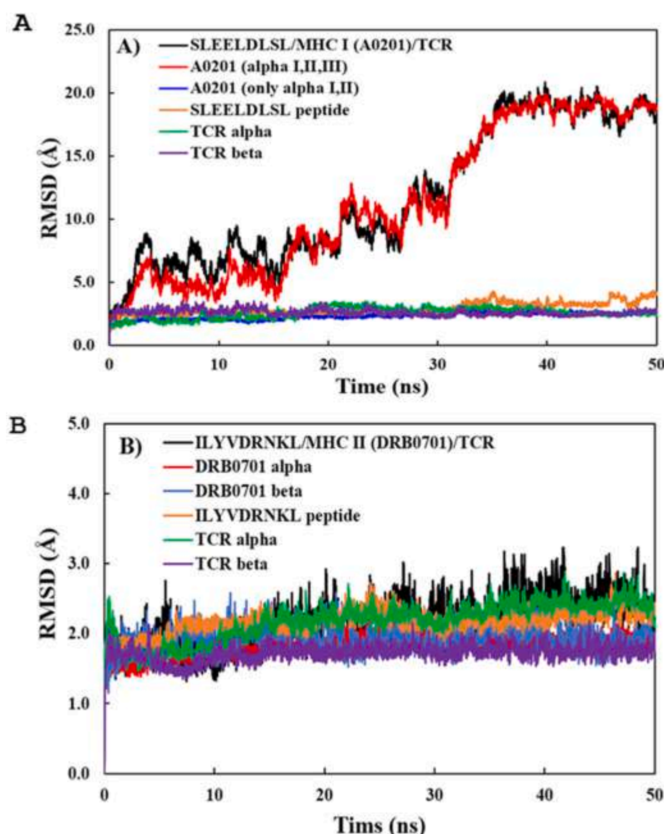

Fig. 2. RMS deviations (Å) of SLEELDLSL/MHCI/TCR (A) and ILYVDRNKL/MHCII/TCR (B) indicating the stability of the entire molecule complex structure were calculated over total simulation time using the initial structure as the reference.

cells than the control group following the third immunization (Fig. 4A and C). Similar results were obtained in the measurement of SL19-specific T cells with a significantly greater frequency of IFNg-producing T cells from the immunized group after the third immunization compared to control group (Fig. 4B). Interestingly, LL17-specific T cells, including CD4$^+$ T cells, exhibited the potent IFNg-producing capacity following the third immunization. However, there was no significant difference in the IFNg production of T cells stimulated with either FN15 or VL17 peptides after immunization in rabbits (data not shown).

3.5. Stimulation of specific humoral immune responses against the rhKU_Sej_LRR_2271 protein in the immunized rabbits

The immunized rabbits were assessed for humoral immune responses by the measurement of specific IgG level in plasma using ELISA. Before and after immunization with the hybrid LRR protein at day 14, 28, and 56, the levels of specific IgG from the immunized and control groups were determined. The results showed that the rabbits immunized with rhKU_Sej_LRR_2271 protein had significantly greater levels of hybrid LRR protein-specific IgG in plasma than that of the control group at day 14, 28 and 56 after immunization as shown in Fig. 5. In addition, the specific antibody levels were increased after the immunization compared with the plasma from control group (*p-value*$\leq$0.05). Increasing of specific humoral immune responses in rabbits immunized with hybrid LRR protein was saturated at day 14 after the second immunization, day 28, until 14 days after the third immunization which was the end of observation on day 56. The result revealed that the rabbits immunized with rhKU_Sej_LRR_2271 protein produced significantly the amount of specific IgG in plasma.

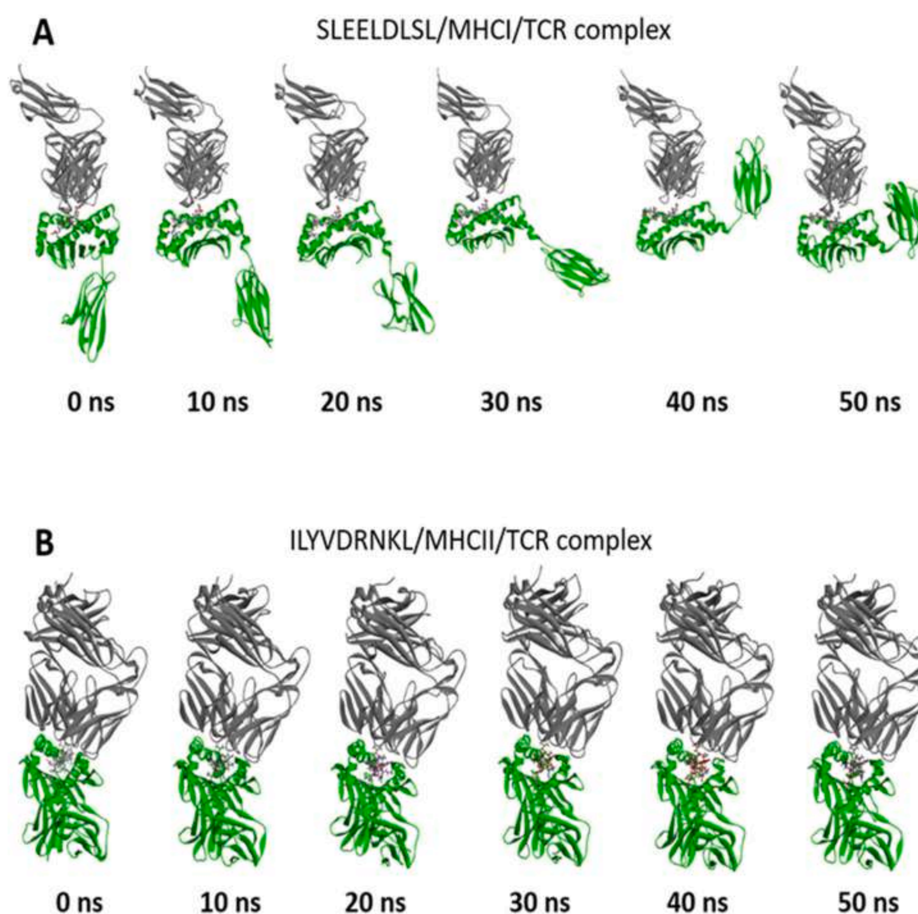

Fig. 3. Snapshots at 0, 10, 20, 30, 40 and 50 ns of (A) SLEELDLSL/MHCI/TCR complex and (B) ILYVDRNKL/MHCII/TCR complex along MD simulations. TCR is shown in grey ribbon, and MHC in green ribbon. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 6
Newly designed peptides containing overlapping epitopes with high prediction score which contain the predicted MHC I and/or MHC II-restricted epitopes and exhibit the highest docking scores.

Peptides	Sequences	No. Of amino acids	Peptide position	Bind to MHC class	Bind to MHC alleles
FN15	FSKLKNLKVLLAGNN	15	148–162	MHCII	DRB* (0101, 0301, 0401, 0701, 1101 and 1501)
LL17	LLFLPLIKILYVDRNKL	17	171–187	MHCI, MHCII	A*0201 and DRB* (0101, 0301, 0401, 0701, 1101, and 1501)
VL17	VEILASLSSLEELDLSL	17	194–210	MHCI, MHCII	A*0201 and DRB* (0101, 0401, 1101, and 1501)
SL19	SLNSGIKALPFNYEKLVNL	19	209–227	MHCI, MHCII	A*0201 and DRB* (0101, and 0701)

4. Discussion

The putative leucine-rich repeat LBJ_2271 ortholog gene in *L. borgpetersenii* serovar Sejroe was previously identified [27] and the recombinant hybrid protein namely rhKU_Sej_LRR_2271 was successfully expressed [4]. Both LBJ_2271 and rhKU_Sej_LRR_2271 proteins were previously predicted as a candidate leptospiral vaccine since they contained the predicted immunogenic epitopes [4,23]. Identification of the immunogenic epitopes can be speedily predicted by computational programs on the World Wide Web (WWW) [10–13,28]. Further investigation of the identified epitopes for the evaluation of immunogenicity is required. Although many methods for the epitope prediction are currently available, each method has its' limited accuracy. The epitope prediction by using the sequence-based analysis alone is not sufficient. Using both sequence and structural based analysis is strongly recommended [13,28–30].

In this study, both sequence and structural based prediction programs were used to identify the epitopes from the rhKU_Sej_LRR_2271 leucine rich repeat protein which could have high immunogenicity for use in leptospiral vaccine development. To strengthen the accuracy of epitope screening, several prediction methods were used. Six algorithms from different programs were used to investigate the potential epitopes based on sequence analysis. Results revealed that the highest number of peptides bound to the most frequent MHC class II alleles is HLA-DRB*0101 allele [31]. The strongest binding affinity of HLA-DRB*0101 allele with epitopes in LBJ_2271 leucine rich repeat protein [23] and the *Leptospira* antigenic protein, LigA [32], was also reported in the previous studies. The predicted epitopes were chosen based on cut-off values and consistency of prediction scores from each method. The predicted epitopes with the highest score from all prediction methods were chosen. We found that the predicted epitopes from the rhKU_Sej_LRR_2271 protein were able to bind to several MHC class I and II alleles. This result indicated the consistency, efficiency and coverage of the prediction.

The molecular docking method was done by ZDOCK and RDOCK protocols [30]. All selected epitopes were modeled to 3D structures and

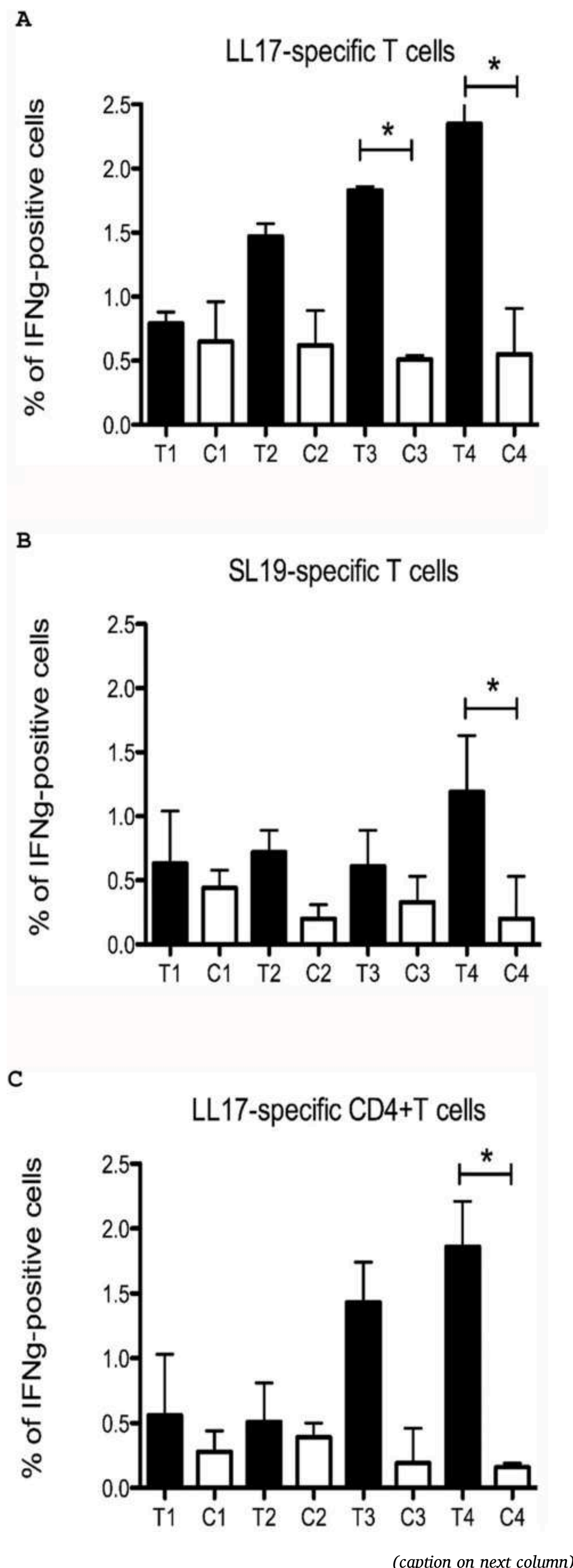

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Fig. 4. *Ex vivo* IFNg-producing T-cell responses against Leptospiral-specific peptides detected by flow cytometry. Percentages of IFNg-producing T-cells responding against **(A)** LL17 peptide and **(B)** SL19 peptide. **(C)** LL17-specific $CD4^{+}$ T-cells were compared between the immunized and control rabbits. The Y axis represents the frequency of intracellular IFNg-positive cells as the percentage of all peripheral blood mononuclear cells. The X axis represents the groups of the immunized (T) and control animals (C). The black bars (T) and white bars (C) represent the immunized ($n = 3$) and the control ($n = 3$) groups, respectively. The 1, 2, 3, and 4 indicate the measurement of peptide-specific T-cell responses before immunization and after the first, the second, and the third immunizations, respectively. The * represents statistically significant differences (*p value* $\leq$ 0.05).

docked on MHC molecules. There are 16 peptides that can form the complex with MHC class II molecules and are recognized by TCR. Only 6 peptides can form the complex with MHC class I molecules. The structure of these docking complexes showed that peptides bind to both MHC class I and II molecules on MHC binding groove and have the interaction with the variable region of TCR. Intermolecular hydrogen bonding was reported between peptide, MHC and TCR structure. The number of hydrogen bonds from the complex structure was nearly similar to the number of hydrogen bonds from the crystal structure [33]. Most epitope residues at the N and C terminal regions have the interaction with MHC and TCR molecules and establish the hydrogen bonds. For the peptide presented on MHC class I molecules, the anchor or hydrophobic residue was previously reported at residue 2, residue at N and C terminus while the auxiliary anchor residue was found at residue 1 and 3 [34]. The docking study showed that residue 1, 2 and 3 are the prominent binding residues according to the number and distance of hydrogen bonds. Residue 1 of peptide on the pMHC class II complex has prominent binding at position 1 which is one of the anchor residue [35]. Docking scores and hydrogen bonds revealed the ability of the candidate peptide as a promising T-cell epitope.

However, not all of the docked peptide-MHC complex can be recognized by TCR in the appropriate orientation as the original PDB structure. Table 4 showed that particular complex has high docking score although the number of hydrogen bonds is very low. Therefore, the low number of hydrogen bonds in the complex structure indicated that the peptide might not reside in the right orientation which affects the recognition of TCR. However, the recognition of pMHC complex by TRC interface required not only hydrogen bonds but also salt bridges, buried surface areas, shape complementarities and van der Waals' contacts [33]. The results indicated the importance of the molecular-based docking study for evaluating an appropriate formation of the peptide-MHC complex.

To emphasize the confidence of the candidate epitope, the structure stability and consistency of the pMHC/TCR complex structure were analyzed by MD simulation. In this study, a 50.0 ns production was run which has been reported for the stable structure in the pMHC/TCR complex [36]. The RMSD was almost stable in the complex of SLEELDLSL/MHC I (A*0201)/TCR after 35 ns of the simulation, while the RMSD fluctuation of the ILYVDRNKL/MHC II (DRB*0701)/TCR complex was quite low and stable along the 50 ns simulations. The fluctuation of RMSD value refers to the flexibility of the structure by which the peptide may not consistently engaged in the binding groove. The flexibility of peptide is induced by exposure of the side chains to the solvent in the unbound state. The flexibility should decrease when the peptide is embedded in the complex of TCR and MHC molecules. These results showed that at least two predicted epitopes, SLEELDLSL and ILYVDRNKL, were competent to be recognized by T cells.

To determine the capability of the 21 predicted T-cell epitopes which have the prediction score above the cut-off value from at least 2 prediction programs, the new peptides were designed based on the high prediction score and/or the ability to bind to MHC class I and II molecules for at least three alleles as shown in Table 6. All four newly designed peptides consisting of FN15, LL17, VL17 and SL19 are likely to

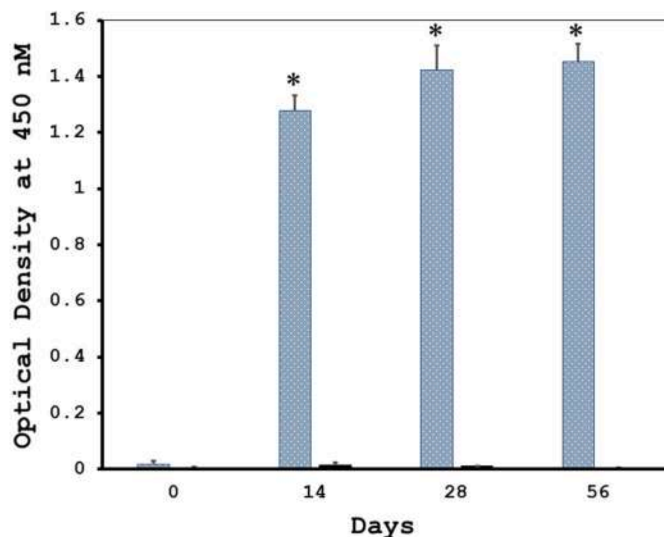

Fig. 5. Induction of specific humoral immune responses in rabbits immunized with recombinant rhKU_Sej_LRR_2271 protein. The rhKU_Sej_LRR_2271-specific IgG was evaluated in rabbit plasma by ELISA. The level of specific IgG production is shown on Y-axis. Plasma from the immunized (Blue bar) and control (Black bar) rabbits were collected before immunization (Day 0) and after immunization on days 14, 28 and 56 as shown on X-axis. The data represent the mean of three rabbits per group $\pm$ S.E.M. The * indicates significant differences (*p value* $\leq$ 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

be the universal immunogenic T-cell epitopes since they are able to stimulate the specific T-cell responses in the immunized rabbits. Two of four newly designed peptides, LL17 and SL19, were highly recognized by T cells from the immunized rabbit group. The rhKU_Sej_LRR_2271 immunized rabbits showed the significantly greater frequencies of IFNg-producing T cells stimulated with LL17 after the third and fourth immunization and high frequencies of IFNg-producing T cells stimulated with SL19 after the last immunization. As for the LL17-specific T cells, the statistically significant frequency of IFNg-producing $CD4^+$ T cells were detected after the third and fourth immunization in the immunized rabbits when compared to the control group.

The results from the stimulation of *ex vivo* LL17 and SL19 specific T cells were comparable to the predicted epitopes derived from the rhKU_Sej_LRR_2271 hybrid protein consisting of 171-LLFLPLIKI, 173-FLPLIKILY, 179-ILYVDRNKL, 209- SLNSGIKAL, and 219-FNYEKLVNL as the potent T-cell epitopes. The highest frequency of IFNg-producing LL17-specific $CD4^+$ T cells was obtained. This peptide contains three predicted epitopes, 171- LLFLPLIKI, 173-FLPLIKILY and 179-ILYVDRNKL, by which the first and the last ones exhibit the highest docking scores and numbers of hydrogen bonding for the pMHC (HLA-DRB*0301, -DRB*0701) and TCR complex structures, respectively. More interestingly, the 171-LLFLPLIKI epitope was able to bind to both MHC class I (A*0201) and class II (DRB*0101, DRB*0301, DRB*0701, DRB*1101 and DRB*1501) alleles. Therefore, the LL17 composition makes the peptide to be the best potent as an *ex vivo* T cells stimulator.

The LL17 peptide can induce the significantly higher numbers of IFNg-producing specific $CD4^+$ T cells in the immunized rabbits compared to the control group. Previous study reported by Volz et al. [37], that IFNg-producing $CD4^+$ T cells from patients with severe infection and mild symptoms were increased after the stimulation with serovars Autumnalis and Bratislava, and with Autumnalis, Bratislava and Copenhageni, at 2–3 weeks and at 4–6 months after infection, respectively. Therefore, these results indicate that the LL17 peptide contains the highly potential to be the target epitope for the stimulation of strong T-cell responses which can be used as the candidate peptide for the development of leptospiral vaccine.

However, the FN15 and VL17 peptides did not show the promising efficacy for the *ex vivo* stimulation of T-cell responses, though the VL17 peptide contains 194-VEILASSLSS and 202-SLEELDLSL epitopes. The latter epitope, though, was proved by molecular docking and simulation studies for the highly recognized of peptide/MHC complex (HLA-A*0201) by TCR. However, by joining epitopes between 194-VEILASSLSS and 202-SLEELDLSL to form the VL17 peptide remarks a pair of serine (S) in the middle of the peptide. The presenting of a serine pair in peptides should be avoided as the conventional wisdom for designing peptides for vaccines [28]. Besides that, the designed peptides were predicted by the bioinformatic tools for overall the binding to human MHC alleles and TCRs rather than to rabbit MHC alleles and TCR, by which the data are not available in the database. Authors performed a multiple sequence alignment between rabbit and human MHC class I and class II sequences as shown in Supplementary Fig. 2. The percentages of the identity created by Clustal 2.1 were 70.17 and 62.01 for MHC class I and class II sequences, respectively. Therefore, the predicted epitopes may not entirely correspond to the rabbit MHC alleles in both MHC class I and class II molecules, and consequently affecting the recognition of rabbit TCR on pMHC complexes.

5. Conclusion

The results from this study demonstrated the success story on using computational bioinformatic tools of *in-silico* sequence and structural based analysis for identifying the potentially promiscuous T-cell epitopes for vaccine candidate. The collaboration between computational analysis and *in vivo* experiment in animal models lead to faster vaccine development. The rhKU_Sej_LRR_2271 hybrid protein was previously predicted as a candidate for leptospiral vaccine since it contains the predicted immunogenic epitopes. In this work, the sequence and structural based analysis were used to evaluate the immunogenicity of rhKU_Sej_LRR_2271. The protein sequence was predicted for the binding affinity of peptides to MHC class I and class II molecules. The predicted epitopes with high binding affinity were modeled to 3D structures to determine the ability of MHC binding and T cell recognition by MD and MDS. Laboratory experiments were also performed to support the ability to be a vaccine candidate. The rabbits immunized with KU_Sej_LRR_2271 recombinant protein showed the profound immunogenicity by the detection of IFNg-producing T-cell responses following the second and the third immunization. The immunogenic peptide, LL17 (171-LLFLPLIKILYVDRNKL-187), has the most promised target epitopes to stimulate cell-mediated immunity. The results indicate that bioinformatics epitope prediction, molecular docking of peptide on MHC, docking of T cell receptor and peptide-MHC complex, and molecular dynamic simulation are powerful tools to be used for vaccine development. *Ex-vivo* study of T-cell responses in animal model using flow cytometry confirmed the accomplishment of the theoretically *in-silico* analysis for identifying the potential T-cell epitopes of the protein. Results indicate that immunization with KU_Sej_LRR_2271 recombinant protein elicits both humoral and cell-mediated immune responses in rabbit model. Therefore, rhKU_Sej_LRR_2271 recombinant protein is a potent protein for leptospirosis vaccine development.

Author contributions

All authors have made substantial contributions to all of the following: SP, YT, PS and TS made the conception and design of the experiments. YT, TS, PS, TB, AC, SS and SP performed the experiments. YT, PS, SH, and SP made acquisition of data, analysis and interpretation of data. YT, PS, TS and SP drafted and wrote the article. SP and SH revised the manuscript critically for important intellectual content. All of the authors read and approved the final version of the manuscript. SP did final approval of the version to be submitted.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imu.2021.100649.

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