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Original Article

The prediction of T- and B-combined epitope of Ag85B antigen of *Mycobacterium tuberculosis*

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Abstract: After decades of intensive control efforts, tuberculosis (TB) still remains one of the most important causes of morbidity and mortality in the world. Antigen Ag85B, a major protein secreted by *Mycobacterium tuberculosis*, is a vaccine candidate against TB. In the present study, we used SignalP, TMHMM, ProPred, BIMAS, SYFPEITHI, DNAStar and IEDB to predict the T- and B-cell epitopes. The analysis of the secondary structure and tertiary structure showed that there were five T-cell epitopes and seven B-cell epitopes. Bioinformatics analysis shows the T- and B-combined epitopes. Four T- and B-combined epitopes that triggering both T cell and B cell responses have been predicted. Four T- and B-combined epitopes were synthetic in rAg85B. The recombinant proteins rAg85B were found to have good immunogenicity. The obtained results in the present study may be beneficial in the investigation of rAg85B antigenicity and the development of dominant epitope vaccines.

Keywords: *Mycobacterium tuberculosis*, Ag85B, epitopes prediction, immunogenicity, vaccines

Introduction

Tuberculosis (TB) is one of the oldest infectious diseases, but it is still the main public health problem in many developing countries of the world [1, 2]. In addition, the emergence of multidrug-resistant TB (MDR-TB) makes the endemic situation even worse [3]. It has been suggested that prevention of TB is the way to reduce MTB cases and vaccine is urgently needed in preventing TB infection.

It has been shown that CD4+ T cells play an essential role in defenses against tuberculosis [4-7] and CD8+ T cells are also involved in the host immune responses to the tuberculosis [8-10]. Therefore identification of T-cell epitopes is important for vaccine development [11-15], including those responsible for CD8+ and CD4+ T cell responses [16, 17].

Recently, increasing evidence has indicated that B cells play crucial roles in MTB immune response, both in antibody dependent and antibody independent way [18-21]. B cells can modulate T cell immunity [22, 23] and shape the development of immune response to *Mycobacterium tuberculosis* [24, 25]. Thus the most effective vaccine should be those which could trigger both T cell and B cell response.

Bacille Calmette-Guérin (BCG) vaccines, one of the most widely used vaccines globally, has 60-80% protective efficacy against severe tuberculosis in children, particularly meningitis [26, 27], but variable efficacy against adult pulmonary tuberculosis and other *mycobacterial tuberculosis* diseases [28, 29]. Hence, the more effective vaccine is most needed. Proteins actively secreted from the live *Mycobacterium tuberculosis* during growth have been shown to improve the protective potency [30], Ag85 complex, the major secretory proteins of *M. tuberculosis*, is a vaccine component in human clinical trials [31]. The Ag85 complex is actually a 30-32 kDa protein family comprised of three related proteins: Ag85A, Ag85B, and Ag85C,

which form Ag85 complex [32, 33]. Ag85B is the dominant component in the complex [34], which stimulates T cells producing IFN-γ and exhibits CTL activity in animal models and in patients infected with *M. tuberculosis TB* [35], making it an excellent candidate of subunit vaccine. Several epitopes for CD4 and CD8 T cells, restricted by different MHC molecules, have been identified [36-38]. In contrast, T-B combined epitopes have very seldom been identified for Ag85B. In present study, we used updated software to predict T-B combined epitopes.

Materials and methods

Animals

7 weeks old BALB/C mice were obtained from Xinjiang Medical University. All mice were used in accordance with animal ethics and experimentation guidelines of First Affiliated Hospital of Xinjiang Medical University. This study was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (approval number: 20120220-126).

Tertiary structure prediction and analysis

Predictive analysis of the Ag85B protein tertiary structure was conducted by online PHYRE2 Protein Fold Recognition Server and Swiss-model (http://swissmodel.expasy.org/interactive). RasMol version software was used to analyze different modes of the tertiary structure. The tertiary structure was displayed in the modes of Cartoon, Structure and Group.

T- and B-combined epitope prediction and analysis

The B-cell epitope prediction software included DNAStar (V5.0) (http://www.dnastar.com) and the online prediction software IEDB (http://tools.immuneepitope.org/main/index.html). The T-cell epitope prediction software included SYFPEITHI (http://www.syfpeithi.de), PROPRED (http://www.imtech.res.in/raghava/propred1/index.html) and BIMAS (http://bimas.dcrt.nih gov/molbiothe/hlabind/).

An epitope is the specific region of protein that can be recognized or band to an antibody and an epitope or an epitope region can be predicated by bioinformatical analysis. Hydrophilicity prediction is based on the calculation of Helmholtz free energy that residues move from organic phase to in organic phase.

Accessibility prediction is corresponded the Distribution of protein antigens, in each layer amino acid.

Antigenicity prediction is based on the antigenicity scale came from the thorough study of consecutive amino acid sites.

Flexibility calculate the activity of Peptide backbone, it is higher possibility to form the epitope when the greater degree of the amino acid amino acid.

All of above features has their threshold, the position appear the peak above the threshold were considered as epitope.

Synthetic of rAg85B

Synthetic of rAg85B that contain epitopes we predicted were carried out by SynPeptide Co Ltd.

Immunization of mice

7 weeks old BALB/C mice were divided into two groups randomly (5 mice each group). (1) rAg85B group: mice were treated with 100 μl of 1×10^9 PFU of rAg85B intranasally three times separated by two-week intervals. (2) Unimmunized group: mice don't receive any treatment. The mice were sacrificed eight weeks later after immunization. Serum and Splenocytes were collected and stored at -80°C until assayed.

Serum Ag85B-specific antibodies

Ag85B-specific antibodies titers were detected by ELISA. Briefly, plates (Becton Dickinson, Oxnard, CA) were coated with Ag85B (50 μl / well) in coating buffer overnight at 4°C. The plates were blocked with phosphate-buffered saline (PBS) containing 5% nonfat milk for 3 h at 37°C and washed four times with PBS containing 0.05% Tween 20. Mice serum samples diluted in 5% skim milk were added in a 50 μl volume to each well and incubated for 1.5 h at 37°C. The plates were washed six times with PBS containing 0.05% Tween 20 and incubated with horse radish peroxidase (HRP)-conjugated goat anti-mouse IgG/IgG1/IgG2a for 1 h at 37°C. The color was developed using tetramethylbenzidine (TMB) solution (Sigma), and

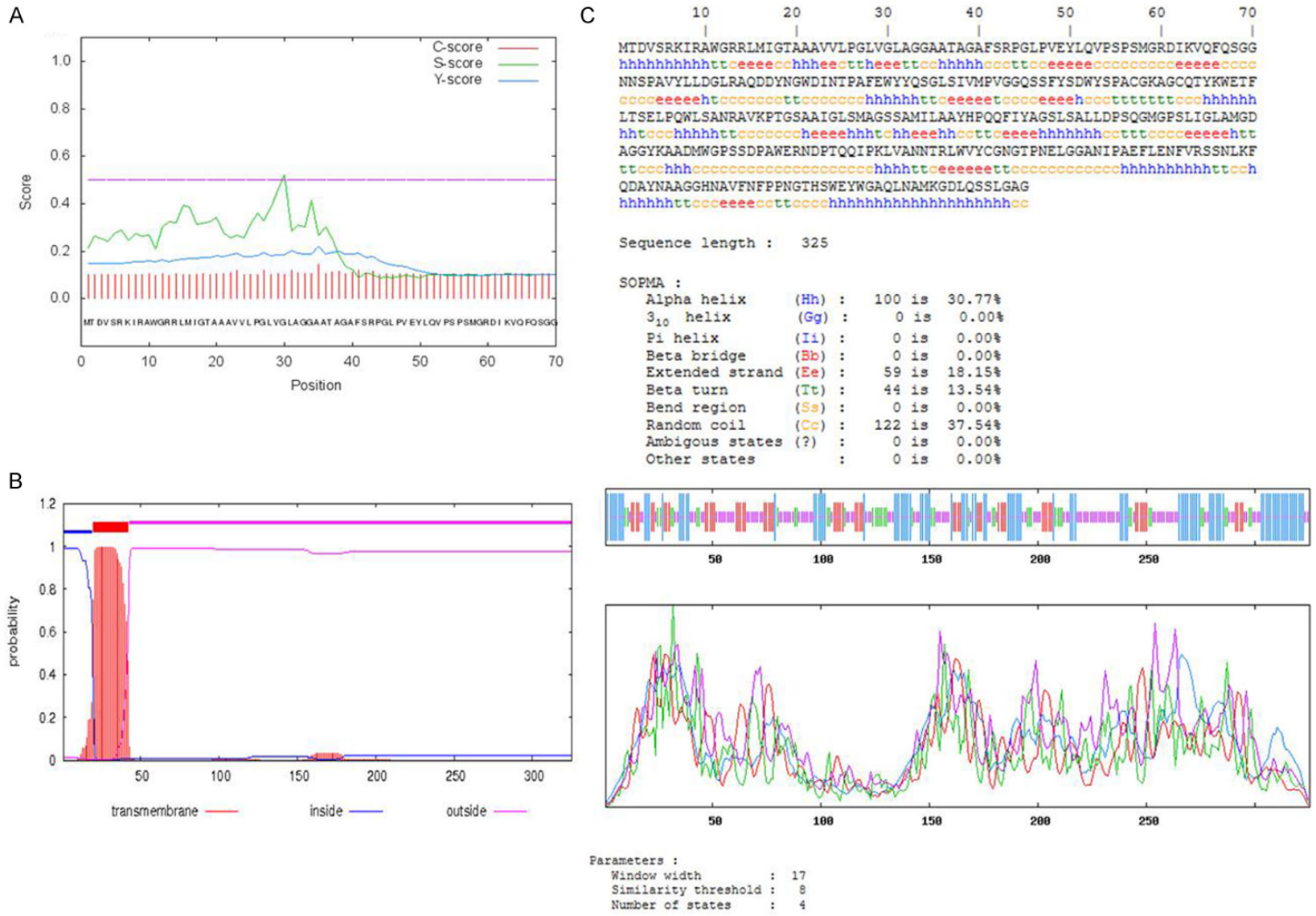


Figure 1. The second structure of Ag85B. (A) Signalp-4.1 Server software was used to analyze the signal peptide. (B) The location of Amino acids were predicted by TMHMM software. (C) The second structure was displayed by SOPMA.

absorbance was measured using an ELISA reader at 450 nm. To determine endpoint titers, serum from unimmunized mice was titrated across an ELISA plate beginning at the same dilution as the samples. The optical density values for each titration point were added together and the average and standard deviation were calculated. The endpoint titer was defined as the mean of the optical densities plus three standard deviations.

IFN-γ ELISPOT

ELISPOT plates were coated with Mouse anti-IFN-γ capture antibody (BD PharMigen, San Diego, CA) diluted 1:60 in PBS overnight at 4°C. The plate then was washed four times with PBST once with PBS. plates were blocked with 100 μl of RPMI-1640 medium containing 10% fetal calf Serum (FCS) for 1 hour at 37°C. Splenocytes were added duplicated to appropriate wells at a final concentration of 1×10^5 per well in a final volume of 100 μl. These cells were stimulated with Ag85B. Con-A at 50 μl per well (10 μg/ml) and medium along at 50 μl per well was used as the positive and negative stimulate respectively. Plates were then incubated at 37°C presents of 5% CO_2 for 24 hours. Cells were removed and washed the plates as described above. 50 μl biotin-labelled secondary antibody (BD PharMigen, San Diego, CA) diluted 1:2000 in 1% PBS was added to each well, plates were incubated at room temperature for 2 hours. Plates were washed and 50 μl streptavidin alkaline phosphatase (Amersham, Life Science, Australia) diluted 1:1000 in 1% BSA was added, and incubated at room temperature for 2 h and the spots were developed by adding BCIP/NBT developer (Moss, USA) to each well for 40 min at room temperature. Plates were washed in water to stop reaction, plate backings were removed and plates were dried at room temperature before counting the spots using an ELISPOT Bio Reader-4000 (BIOSYS, GmbH, Germany).

Result

The amino acid sequence of Ag85B antigen which contained 325 amino acids obtained from Genbank, it listed below:

MTDVSRKIRAWGRRLMIGTAAAVVLPGLVGLAGGAATAGAFSRPGLPVEYLQVPSPSMGRDIKVQFQSGGNNSPAVYLLDGLRAQDDYNGWDINTPAFEWYYQSGLSIVMPVGGQSSFYSDWYSPACGKAGCQTYKWETFLTSELPQWLSANRAVKPTGSAAIGLSMAGSSAMILAAYHPQQFIYAGSLSALLDPSQGMGPSLIGLAMGDAGGYKAADMWGPSSDPAWERNDPTQQIPKLVANNTRLWVYCGNGTPNELGGANIPAEFLENFVRSSNLKFQDAYNAAGGHNAVFNFPPNGTHSWEYWGAQLNAMKGDLQSSLGAG.

Prediction of secondary structure of Ag85B

SignalP-4.1 was applied to predict the signal peptide. The result predicted was list (**Figure 1A**). S score is the signal peptide score, which suggesting the position of signal peptide. C score should combined Y score to predict the signal peptide. The result predicted by three score was better than that predicted by the score single. We speculated that there is no signal peptide in Ag85B. Transmembrane and nontransmembrane region of Ag85B were predicted by TMHMM software. The program predicated that the sequence region of the first 19 amino acids is intracellular region. The amino acid starts from 20 to 42 are transmembrance. The region of amino acids start from 43 to 325 are extracellular domain (**Figure 1B**). The secondary structure analyses of Ag85B protein was carried out by SOPMA. We found the flexible structure is 69.23%, Extended strand is 18.15%, Beta turn 13.54%, Random coil is 37.54% (**Figure 1C**). Flexible structure is the areas could distortion which easy to combine with antibodies. Alpha helix is 30.77%, these areas are difficult to change the structure, and therefore Alpha helix generally does not used as epitope.

Tertiary structure prediction results of Ag85B protein

To gain a better understanding of its conformational structure, the tertiary structure of Ag85B was analyzed by Swiss-model analysis. In Swiss-model, Subsequent to minor adjustments by the RasMol version software, the tertiary structure predicted by Swiss-model were identified (**Figure 2**). Striking feature of Ag85B is the disordered N-, middle and particularly C-termini of proteins. In the middle of the protein structure, a lot of α helixes were identified. These structures showed that the Ag85B protein had litter spatial flexibility and scalability.

T cell epitope prediction of Ag85B

In order to improve the accuracy of prediction, we predicted the T cell epitope with ProPred,

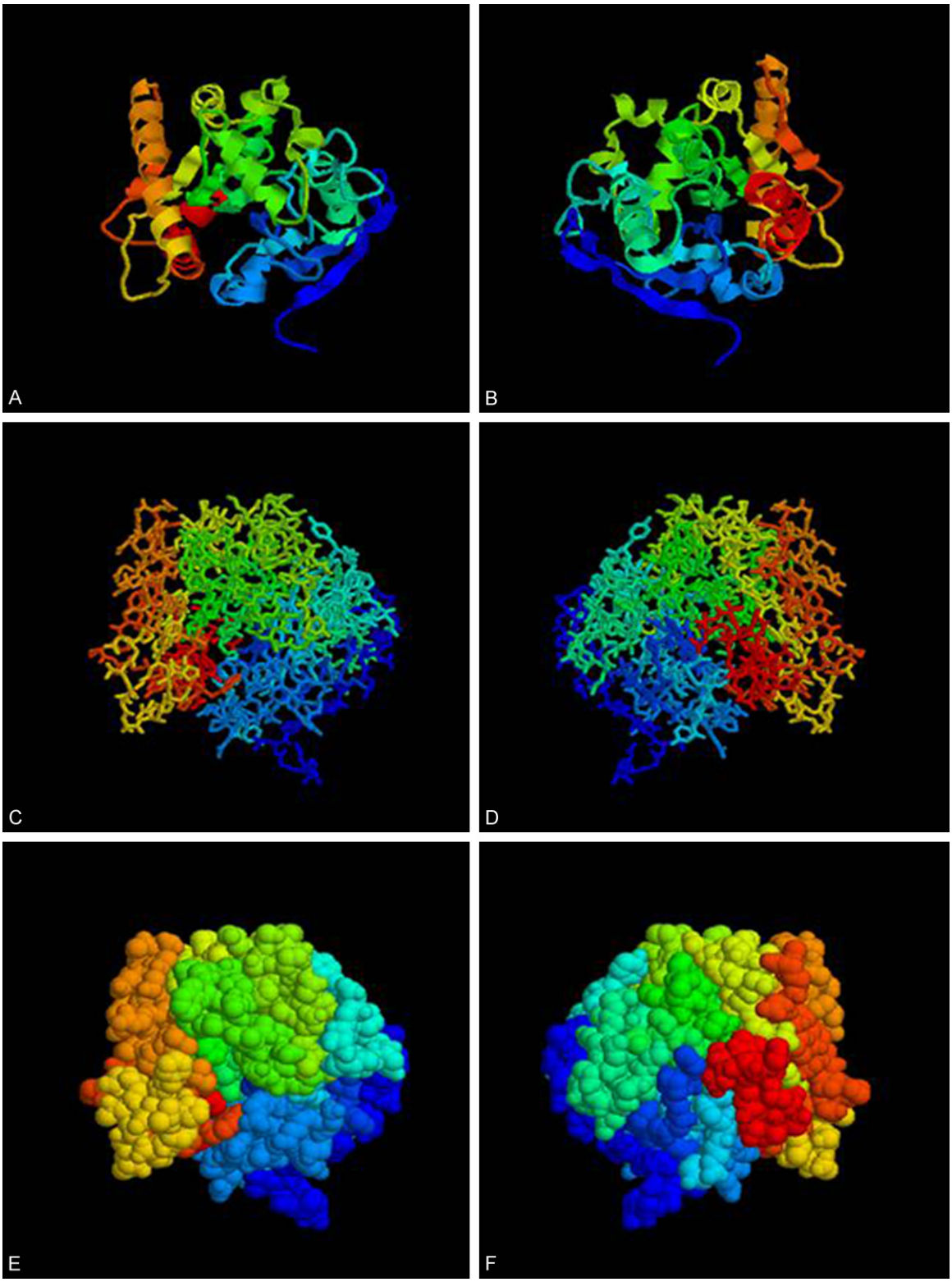


Figure 2. Tertiary structure prediction of Ag85B. The structure of the protein was modeled using Phyre, α helixes are shown by the curved lines and the disorder are shown by the thread let. (A) The front view (B) the back view. The Tertiary structure of the Ag85B modeled using Phyre, analysed by Rasmol software in srick and group (C) the front view (D) the back view. The Tertiary structure of the Ag85B modeled using Phyre, analysed by Rasmol software in space fill and structure .magenta area corresponded to the α helixes, pale blue area corresponded to the turns. White area corresponded to the other (E) the front view (F) the back view.

Table 1. T cell epitopes in the Context of HLA-A*0201

ProPred			BIMAS			SYFPEITHI		
Position	Sequence	Score	Position	Sequence	Score	Position	Sequence	Score
45	GLPVEYLQV	104.33	45	GLPVEYLQV	104.328	23	VVLPGLVGL	28
15	LMIGTAAAV	85.4	15	LMIGTAAAV	85.394	15	LMGTAAAV	27
191	ALLDPSQGM	75.36	191	ALLDPSQGM	75.365	198	GMGPSLIGL	26
239	KLVANNTRL	74.77	239	KLVANNTRL	74.768	30	GLAGGAATA	25
198	GMGPSLIGL	35.49	198	GMGPSLIGL	35.485	20	AAAVVLPGL	24
23	VVLPGLVGL	27.04	23	VVLPGLVGL	27.042	166	SMAGSSAMI	24
50	YLQVPSPSM	22.85	50	YLQVPSPSM	22.853	14	RLMIGTAAA	22
14	RLMIGTAAA	18.38	14	RLMIGTAAA	18.382	24	VLPGLVGLA	22
98	FEWYYQSGL	15.86	98	FEWYYQSGL	15.859	45	GLPVEYLQV	22
141	LTSELPQWL	11.37	141	LTSELPQWL	11.374	77	YLLDGLRAQ	22

Table 2. T cell epitopes in the Context of HLA-DR*0301

ProPred			BIMAS			SYFPEITHI		
Position	Sequence	Score	Position	Sequence	Score	Position	Sequence	Score
57	MGRDIKVQF	5.9	57	MGRDIKVQF	5.9	311	LNAMKGDLQSSLGAG	36
14	LMIGTAAAV	3.4	14	LMIGTAAAV	3.4	55	SPSMGRDIKVQFQSG	35
162	IGLSMAGSS	3.4	162	IGLSMAGSS	3.4	269	LENFVRSSNLKFQDA	26
313	MKGDLQSSL	3.33	313	MKGDLQSSL	3.33	107	SIVMPVGGQSSFYSD	25
23	VLPGLVGLA	3.1	23	VLPGLVGLA	3.1	20	AAAVVLPGLVGLAGG	22
107	IVMPVGGQS	3	107	IVMPVGGQS	3	95	TPAFEWYYQSGLSIV	22
271	FVRSSNLKF	3	271	FVRSSNLKF	3	138	ETFLTSELPQWLSAN	22
47	VEYLQVPSP	2.5	47	VEYLQVPSP	2.5	75	AVYLLDGLRAQDDYN	20
22	VVLPGLVGL	2.46	22	VVLPGLVGL	2.46	181	QQFIYAGSLSALLDP	20
7	IRAWGRRLM	2.4	7	IRAWGRRLM	2.4	5	SRKIRAWGRRLMIGT	19

BIMAS and SYFPEITHI. HLA molecule type selected: HLAA_0201 (**Table 1**) and HLADR_0301 (**Table 2**). The epitopes predicated by these software were the ultimately defined as the T cell epitopes. The T cell Epitope positions of Ag85B were 45-58, 77-88, 98-107, 191-206.

B cell Epitope prediction

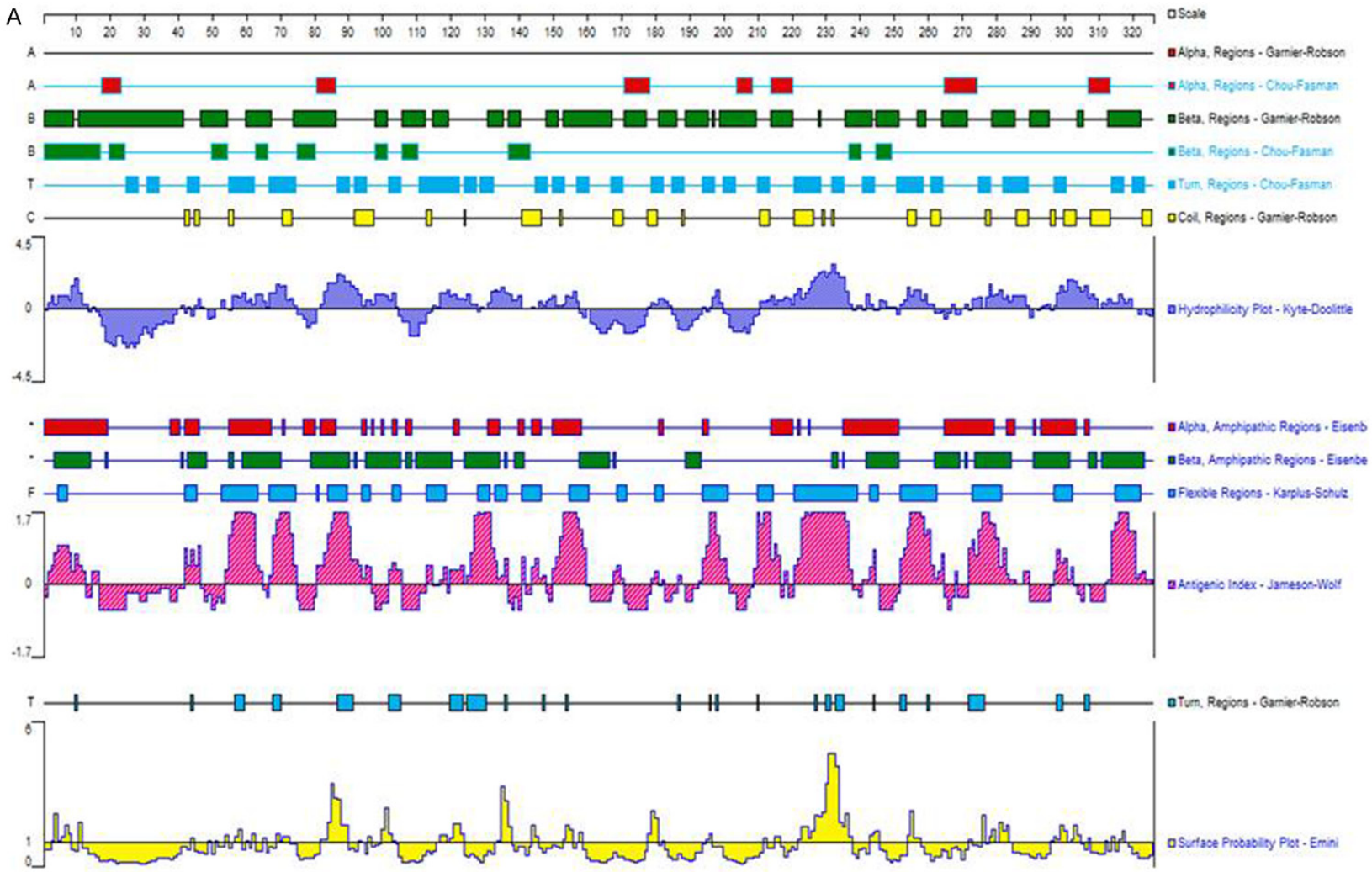
We predicted the B cell Epitope with DNAstar and IEDB software.

The results calculated according to the different features in the IEDB software (**Figure 3**). There is a great probability that the position appear the peak above the threshold could be the epitope. Beta-Turn Prediction: the highest score position is 68-74, next are 67-73, 251-257, 220-226, 41-47, high score position mainly distributed in the range of 41-51. Surface Accessibility Prediction: the score of position 30-35 is 3.458, which is the highest. The next are 2-7, 33-38, 55-60, 56-61. Antigenicity prediction: the top five are 73-79, 47-53, 48-54, 106-112. Flexibility prediction: the top five positions are 68-74, 67-73, 69-75, 66-72, 112-118. The top five positions of Hydrophilicity prediction are 67-73, 229-235, 68-74, 230-236, 252-258.

The B cell Epitope predictions of Ag85B were defined by analyzing the common position predicted by DNAstar and IEDB software. The B cell Epitope positions of Ag85B were 83-92, 220-228, 250-259, 295-305 (**Table 3**).

T- and B-union epitope prediction results of Ag85B

Based on the tertiary structure of Ag85B antigen, we predicted the potential T- and B-combined epitopes of Ag85B antigen. The T-cell epitope was predicted by the SYFPEITHI and


A
Scale
Alpha, Regions - Garnier-Robson
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Beta, Regions - Garnier-Robson
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Coil, Regions - Garnier-Robson
Hydrophilicity Plot - Kyte-Doolittle
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Antigenic Index - Jameson-Wolf
Turn, Regions - Garnier-Robson
Surface Probability Plot - Emini

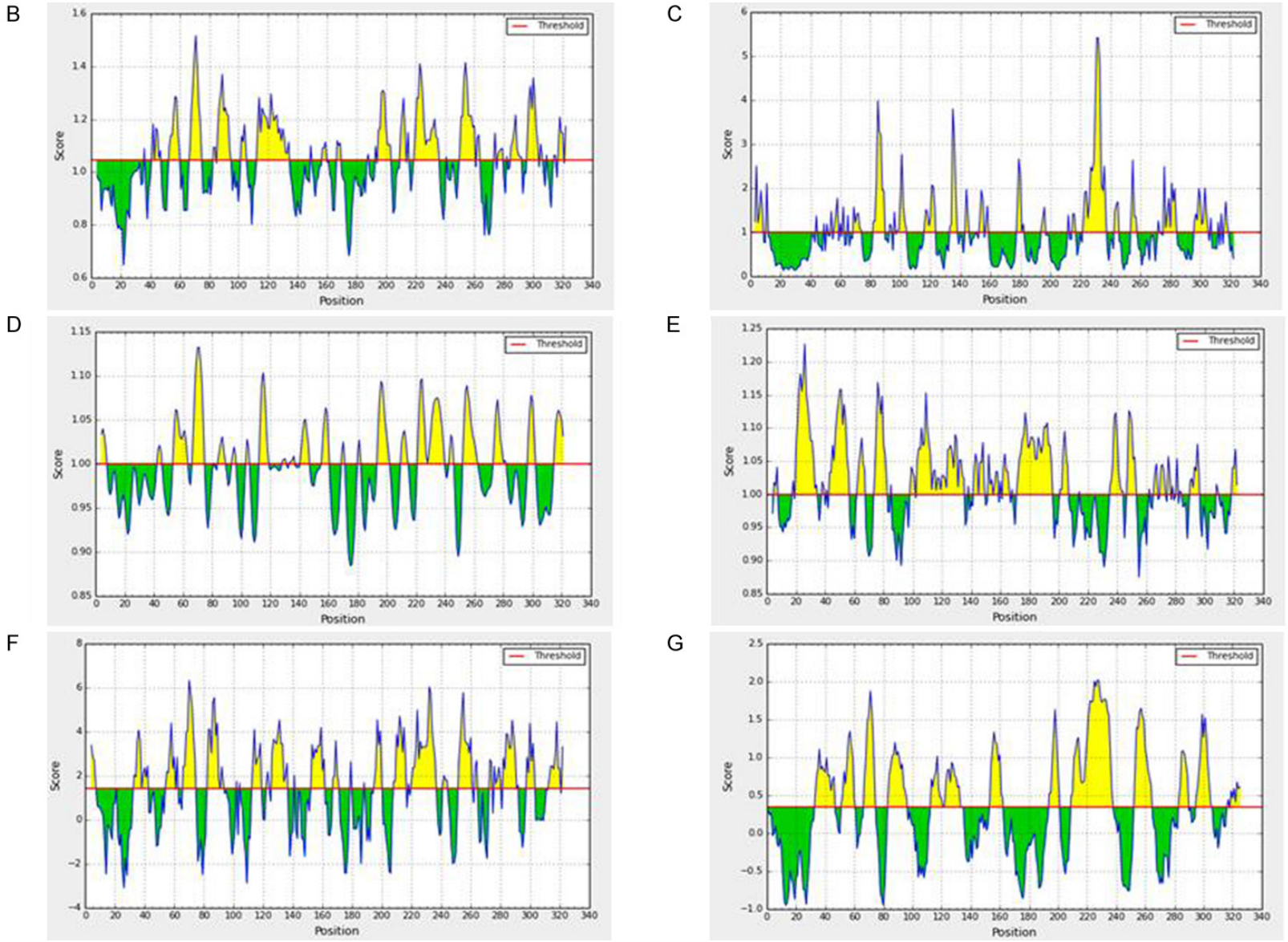

Figure 3. B cell Epitopes prediction. (A) B cell Epitopes predicted by DNAStar. (B) IEDB online software was used to predict the B cell Epitopes. Chou & Fasman Beta-Turn Prediction, the threshold is 1.045. (C) Emini Surface Accessibility Prediction, the threshold is 1.0 Minimum. (D) Karplus & Schulz Flexibility Prediction, the threshold is 1.0. (E) Kolaskar & Tongaonkar Antigenicity Prediction, the threshold is 1.0. (F) Parker Hydrophilicity Prediction, the threshold is 1.414. (G) Bepipred Linear Epitope Prediction, the threshold is 0.35.

Table 3. The T-B cell epitope prediction

Epitope	Position	Sequence
B cell epitope	46-61	LPVEYLQVPSPSMGRD
	65-92	QFQSGGNNSPAVYLLDGLRAQDDYNGWD
	106-125	LSIVMPVGGQSSFYSDWYSP
	192-202	LLDPSQGMGPS
	220-238	WGPSSDPAWERNDPTQQIP
	250-259	CGNGTPNEL
	295-303	FPPNGTHS
T cell epitope	5-38	SRKIRAWGRRLMIGTAAAVVLPGLVGLAGGAATA
	45-69	GLPVEYLQVPSPSMGRDIKVQFQSG
	75-89	VYLLDGLRAQDDYN
	95-129	PAFEWYYQSGLSIVMPVGGQSSFYSDWYSPACGK
	191-206	ALLDPSQGMGPSLIGL
T-B union epitope	46-61	LPVEYLQVPSPSMGRD
	75-89	VYLLDGLRAQDDYN
	106-125	LSIVMPVGGQSSFYSDWYSP
	192-202	LDPSQGMGPS

BIMAS software, while the B-cell epitope was predicted by software included DNAStar software and the online prediction software IEDB. The prediction results are shown in **Table 1**, with the T epitopes in bold. The overlapping regions of T- and B-cell epitopes formed the T- and B-combined epitopes. We find the T-B union epitopes are 46-61, 75-89, 106-125, 192-202 (**Table 3**).

Then, Ag85B-1 contain predicted epitopes 46-61, 75-89. Ag85B-2 contain predicted epitopes 106-125, 192-202.

Antibody specific humoral response induced by rAg85B immunization

In this study, we evaluate antibody response induced by rAg85B. We examined the Ag85B-specific antibody titers of IgG, IgG1 and IgG2a. Antibody titers of mice serum against Ag85B were detected with ELISA. There is significant difference of IgG titers between the rAg85B immunized mice and unimmunized mice. Significantly elevated IgG was found in rAg85B immunized mice. We determined the IgG2a and IgG1 in order to evaluate the immunize response triggered by rAg85B. There is significant difference of the IgG2a/IgG1 ratio between the rAg85B immunized mice and unimmunized mice. IgG2a/ IgG1 ratio of rAg85B group was higher than that of unimmunized Group.

Th1 immunize response induced by rAg85B immunization

In order to confirm that rAg85B could induce the Th1 response, we evaluated the IFN-γ response after rAg85B vaccination. IFN-γ secreting T cells from Splenocytes were detected by ELISPOT. It is found that there are more IFN-γ secreting T cells in Splenocytes from immunized mice with rAg85B than unimmunized mice. As result, rAg85B exhibit the strong immunogenicity.

Discussion

The aim of the present study focused on the prediction of T-B combined epitopes derived from Ag85B. The T-B combined epitopes were predicted with epitope predict tools. The T-B combined epitopes were 46-61, 75-89, 106-125, 192-202.

Cellular immunity plays a critical role in controlling *M. tuberculosis* infection [39, 40]. CD4+ T cell is believed as the essential component of protective immunity against tuberculosis [41]. CD4+ T cells recognize *M. tuberculosis* antigens presents by major histocompatibility complex (MHC) Class II molecules on antigen presenting cells, then specific CD4+ T cells secrete the macrophage activating cytokines IFN-γ and TNFa/b, which help macrophages control intracellular mycobacteria [42]. Antigen recognition by CD4+ T cells is tightly restricted by MHC Class II genes (HLA). Some studies demonstrated that pare of epitopes could be recognized by CD4+ T cells in the context of at least in DRB1*0401, DRB5*0101, and DQB1*0302 [43], the allele DRB1*0301 is a major class II allele that is present in 20% of the human population. HLA-DR3 is associated with high-responder tuberculoid and with strong T cell activity to mycobacterial Ags [44], hence we study the epitopes of CD4+ T cells in the context of HLA-DRB1*0301.

The study for CD8+ T cells is not quite compelling, however, some studies suggested that

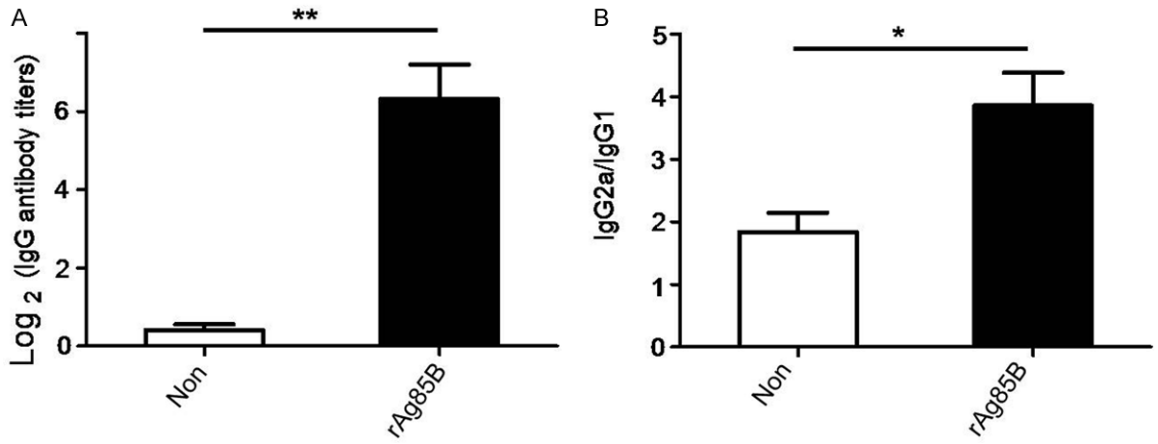

Figure 4. Serum Ag85B-specific antibodies. Antibody against Ag85B in immunized mice. Mice was sacrificed and serum were obtained after four weeks immunized with rAg85B and unimmunized mice. IgG and IgG1 and IgG2a against Ag85B were detected with ELISA. (A) rAg85B immunization response show an increase IgG response to Ag85B. (B) rAg85B immunized mice exhibit the higher ratio of IgG2a/IgG1 than that of unimmunized mice. Data were expressed as Mean ± SEM. Non: unimmunized mice; rAg85B: mice immunized with rAg85B. *$P < 0.05$, **$P < 0.01$.

human CD8+ T cells recognize *M. tuberculosis* infected macrophages had the ability to directly kill intracellular mycobacteria [45, 46]. So that the MHC-class I-restricted CD8+ T cells have been highly paid attention. HLA-A*0201 is one of the most prevalent MHC-I alleles, with the frequency over 30% in most populations [47], thus the study of HLA-A*0201-binding epitope derived from Ag85B is very important. CD8+ T cells are associated with strong CD4+ T cell responses [48], A strong CD4+ T cell response is induced and is necessary, but apparently not sufficient to deal an infection [49-51]. Is that sufficient for protection against TB by triggering the effective of CD4+ T cell and CD8+ T cell? Emerging experimental evidence suggests that B cells play a role in defense against a wide variety of intracellular bacterial, B and T cell responses function complementarily to repel natural infection, and can likewise both contribute to long-lived protection as the result of vaccination [52, 53]. So that vaccine generating both T cells and B cells response are the one optimal protection against TB.

Ag85B has no signal peptide but contain amino acid in the membrane and transmembrane parts which can't be presented to T cell by MHC molecules and are not able to bind BCR, the epitopes therefore should be the parts of amino acid between 43 to 325, (**Figure 1A**, **1B**). In order to improve the confidence level of the prediction results, the overlap epitopes predicted by ProPred, BIMAS and SYFPEITHI online analysis software were defined as the T cell epitopes.

We predicted the second structure and tertiary structure of Ag85B. SOMPA method was used to predict the second structure. We found that the most part of Ag85B were flexible structure (**Figure 1C**), which offer the precondition for forming antigen epitopes. Flexible structure is the distortion areas, which is easy to combine with antibodies. While the epitopes are usually located on the surface of the molecule and have good hydrophilicity, so that the flexible structure on the surface of molecule would turn into the antigen epitopes with great possibilities. We predicted the tertiary structure of Ag85B based on the I-TASSER model and Phyre model. I-TASSER usually provides the first and most reliable template parameters, C score is used for estimating the quality of predicted models, which is calculated based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C score is usually in the range of -5 to 2. The high value suggests the high confidence. TM-score and RMSD are used to evaluate the structural similarity between the two structures [54, 55]. In present study, the C score is -0.03, the Exp. TM-Score is 0.71± 0.12, the Exp. MSD is 6.4±3.9. Usually, it is meaningful when TM-Score is more than 0.5, the accessed tertiary structure is the one which

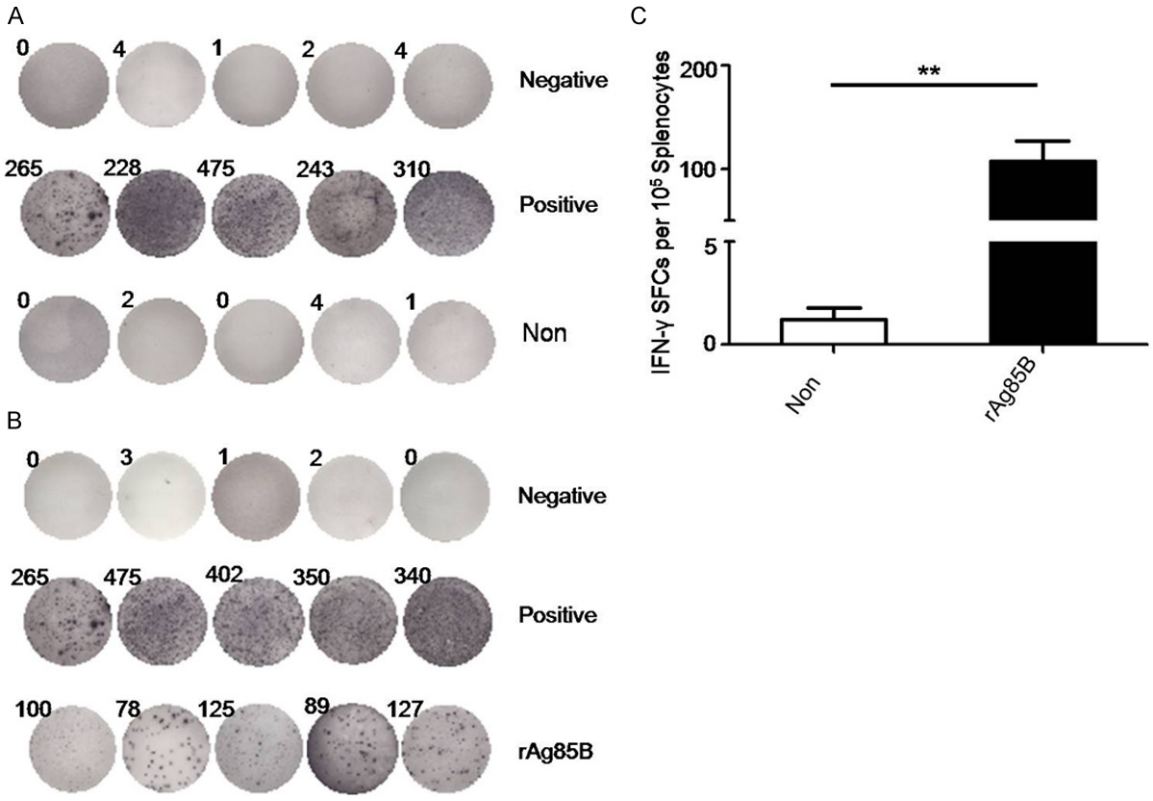

Figure 5. IFN-γ ELISPOT assay. Frequency of IFN-γ secreting T cells in Splenocytes of immunized mice. Immunized Mice were sacrificed at four week for Splenocytes separation. T cell from Splenocytes was stimulated with Ag85B. (A) IFN-γ secreting T cells in Splenocytes from unimmunized mice. Splenocytes were stimulated with Ag85B. (B) IFN-γ secreting T cells in Splenocytes from mice immunized with rAg85B. (C) Frequency of IFN-γ secreting T cells in mice immunized with rAg85B is much higher than that of mice unimmunized. Negative: Splenocytes weren't stimulated. Positive: Splenocytes were stimulated with ConA. rAg85B: Splenocytes from mice immunized with rAg85B. SFU: spot-form units. $*P < 0.05$, $**P < 0.01$.

is closest to its natural conformation through bioinformatics prediction.

Epitopes can be divided into T- and B-cell epitopes. T cells and B-cells can recognize the T- and B-cell combine epitopes at the same time. Theory, the vaccine containing the T- and B-cell joint epitopes has much more stronger protection than the one that containing the T- or B-cell epitopes only. In present study, we synthetic the rAg85B contain sequences of four T- and B-cell combined epitopes.

Th1 and Th2 immune response is different in *M. tuberculosis* infection. Cell mediated immunity response is triggered by Th1 response, which produce IgG2a antibodies. Th2 immune responses induce humoral immunity characterized by IgG1 immune response. To verify the immune characteristics of rAg85B, we detected the IFN-γ secreting T cells of Splenocytes from immunized mice with rAg85B as well as Ag85B-special IgG and the ratio of IgG2a/IgG1. It is exciting to found that the level of IgG titer and the ratio of IgG2a/IgG1 in the mice immunized with rAg85B is much higher than that of mice unimmunized (**Figure 4**). Much more IFN-γ secreting T cells from Splenocytes in rAg85B immunized mice is also observed (**Figure 5**). These results suggest that rAg85B is able to trigger cell-mediated immune response and elicit strong humoral immune response.

Therefore the T- and B-cell combined epitopes we predicted in present study can be used as new vaccine candidate which can reduce the infection risk of *Mycobacterium tuberculosis.* Furthermore, it provides a new way for the development of new effective vaccines and lays the experimental foundation for prevention of Mycobacterium tuberculosis infection.

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Disclosure of conflict of interest

None.

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