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Selection of *SARS-Coronavirus*-specific B cell epitopes by phage peptide library screening and evaluation of the immunological effect of epitope-based peptides on mice☆

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Abstract

Antibodies to *SARS-Coronavirus* (*SARS-CoV*)-specific B cell epitopes might recognize the pathogen and interrupt its adherence to and penetration of host cells. Hence, these epitopes could be useful for diagnosis and as vaccine constituents. Using the phage-displayed peptide library screening method and purified Fab fragments of immunoglobulin G (IgG Fab) from normal human sera and convalescent sera from *SARS-CoV*-infected patients as targets, 11 B cell epitopes of *SARS-CoV* spike glycoprotein (S protein) and membrane protein (M protein) were screened. After a bioinformatics tool was used to analyze these epitopes, four epitope-based S protein dodecapeptides corresponding to the predominant epitopes were chosen for synthesis. Their antigenic specificities and immunogenicities were studied *in vitro* and *in vivo*. Flow cytometry and ELISPOT analysis of lymphocytes as well as a serologic analysis of antibody showed that these peptides could trigger a rapid, highly effective, and relatively safe immune response in BALB/c mice. These findings might aid development of SARS diagnostics and vaccines. Moreover, the role of S and M proteins as important surface antigens is confirmed.

Keywords: SARS; B cell epitope; Phage display peptide library; Peptide; Flow cytometry; ELISPOT

Introduction

Between 2002 and 2003, Severe Acute Respiratory Syndrome (SARS), a new virus infectious disease with high fatality rate, spread to 32 countries. A novel coronavirus designated "*SARS-Coronavirus (SARS-CoV)*" was responsible for the serious infectious disease (Drosten et al., 2003; Fouchier et al., 2003; Ksiazek et al., 2003; Peiris et al., 2003; Poutanen et al., 2003). Although the global outbreak of SARS was controlled and the virus had been isolated from wild animals (layan palm civets and raccoon dogs), the actual natural reservoir for *SARS-CoV* is still unknown and its reemergence in the future remains a possibility. To better prevent future SARS epidemics, protective vaccines and reliable diagnostic reagents must be developed to control this life-threatening disease.

B cell epitopes are defined as regions on the surface of the native antigen that are recognized and bind to B cell receptors or specific antibodies. These epitopes are the focus of immunological research as well as the targets of development of vaccine and diagnostic reagent (Vanniasinkam et al., 2001; Viudes et al., 2001). Therefore, in the present study, we screened and identified specific B cell epitopes of *SARS-CoV* using phage-displayed peptide library, Fab fragments from anti-*SARS-CoV* immunoglobulin G (IgG) and normal human IgG as targets, and an improved biopanning procedure. The immune responses induced by the four epitope-based peptides were also evaluated with animal experiments.

☆ This work was performed in laboratories of the Department of Microbiology, Zhongshan Medical School, Sun Yat-sen University.

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Results

Immunoselection of B cell epitopes

The product of serum treatment, anti-*SARS-CoV* IgG Fab (45 kDa) was shown by ELISA and Western blotting to bind specifically to *SARS-CoV* antigen. The immunoscreening with the two IgG Fab targets enriched the phage population binding to anti-*SARS-CoV* IgG Fab. Of 60 selected phage clones, 43 (72%) immunopositive clones had significant enhancement of binding activity to *SARS-CoV*-specific antibodies but not to normal human serum (Fig. 1). The homology between peptide sequences of a series of epitopes inserted into target phages and the primary sequence of the structural protein of *SARS-CoV* was compared (Table 1). As seen in Table 1, the double selection process resulted in 12 different peptides, which could be divided into three groups: (1) nine peptides with homology to nine different regions of the *SARS-CoV* S protein; (2) two peptides with homology to two regions of the M protein; and (3) one peptide without any homology to the *SARS-CoV* structural proteins. Some peptides showed a striking resemblance (as its peptide sequence SHVPLATSRTLA contained six matches to the M protein sequence) and were isolated more than once (SHVPLATSRTLA was sequenced five times, Group 2 in Table 1). The majority of epitopes were isolated after both screens.

After general evaluation of these epitopes with bioinformation software, four predominant epitopes of *SARS-CoV* S protein were selected and the corresponding epitope-based peptides (MAP1, ^{91}ATEKSNVVRGWV^{102}; MAP2, ^{424}NTRNIDATSTGN^{435}; MAP3: ^{458}PFSPDGKPCTPP^{469}; and MAP4, ^{1065}HEGKAYFPREGV^{1076} [according to AAT74874]) were synthesized. To prove that the phage-borne peptides were the real epitopes of *SARS-CoV*, a peptide competitive-inhibition assay was performed to determine whether the synthetic peptide and the corresponding phage clone competed for the same antibody-binding site. In competitive-inhibition assay, the peptides dose-dependently blocked the binding of the corresponding phage clones to the targets (Fig. 2). Moreover, the four peptides reacted with *SARS-CoV*-specific serum antibody and did not cross-react with anti-*HCoV-229E* and *HCoV-OC43* sera or healthy donor sera (Fig. 3).

Immunological effect elicited by MAPs

The antibody responses elicited by the epitope-based peptides were determined using ELISA. Titers of the antisera from challenged mice are shown in Fig. 4. Every epitope-based peptide stimulated antibody production, even after just primary immunization. A second immunization substantially increased antibody titer within a week. The specific antibody was still detectable on day 42 with high titer suggesting that a strong and relatively long-term humoral immune response was induced by each of the four B cell epitopes. These anti-peptide antibodies reacted with *SARS-CoV* antigen specifically but failed to react with *HCoV-229E* and *HCoV-OC43* antigens (Fig. 5).

The effects of these peptides on splenic lymphocyte subsets isolated from challenged mice were analyzed using two-color flow cytometry. On day 14 or day 21 after primary immunization, the relative numbers of $CD3^{-}CD19^{+}$ B cells increased significantly and that of $CD3^{+}$ T cells decreased relative to control group levels (Table 2). Although both the number of $CD4^{+}$ T cells and $CD8^{+}$ T cells decreased simultaneously, the decrease was greater in the latter than the former. Consequently,

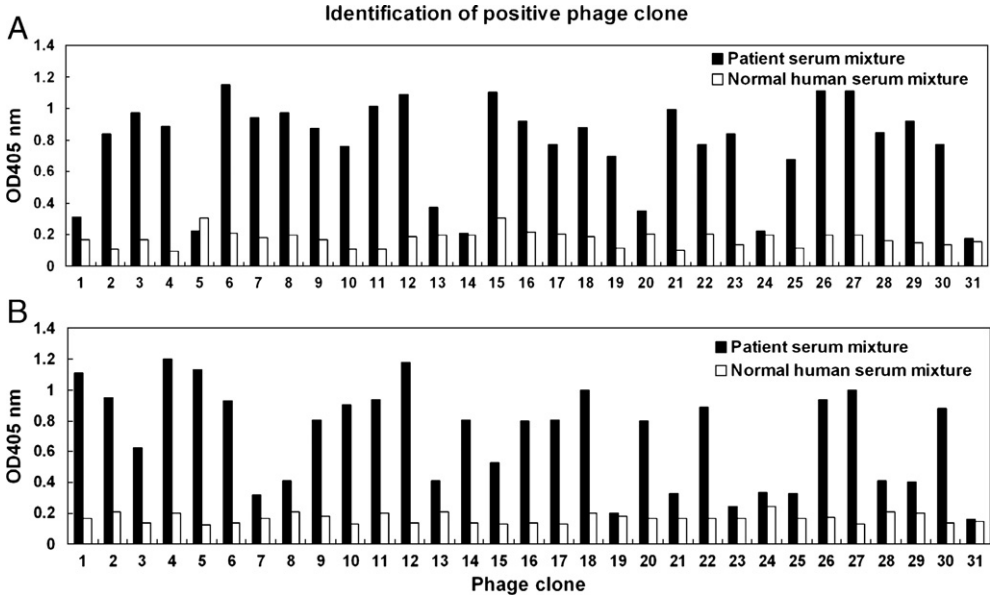

Fig. 1. Identification of specific phage clones reactive with anti-SARS-CoV serum by ELISA. 1–30 represent the ELISA results reacted between different phage clones selected by immunoscreen and patient serum mixture/normal human serum mixture. 31 represent the results between wild type phages as negative control and serum samples above. After four rounds of biopanning twice, 43 phage clones from 60 selected phage clones were significantly reactive with anti-SARS-CoV serum, but not with normal human serum. A and B represent respectively the immune reactivity of phage clones picked at random from the first and the second time that phage cloning was carried out. The negative control reacted with neither patient serum mixture nor normal human serum mixture.

Table 1
SARS-Cov sequence aligned and compared with deduced dodecapeptide sequences of the phage peptides isolated by two separate screens of the phage library

		Frequency
Group 1		
S protein	^{88}Y F A A ***T E*** K S N ***V*** V ***R***^{99}	
	^{a}N L P S **T E** T R H **V** T **R**	5 (1, 2)
S protein	^{133}C D N ***P*** F F A ***V S*** K ***P*** M G T Q^{147}	
	V F G **P** S Y N **V** K H **P** T **G**	1 (1)
	P F A E C **S** N **P** T R L P	1 (2)
S protein	^{421}L A W N ***T R*** N I D ***A*** T ***S*** T G N Y^{436}	
	M I Y S **T** Q P F E **A S S**	1 (1)
	^{a}**T R** Y N**D A** L **S** H D R L	1 (2)
S protein	^{458}V P ***F S P D*** G K ***P*** C T P ***P*** A ***L*** N^{473}	
	H H R P **P** S H T **P** T **L** F	2 (1, 2)
	^{a}H H **F S** S **D** P R**P** D L H	2 (1, 2)
	D **P F F P** W P R T T F D	1 (2)
S protein	^{532}***L T G T*** G V L T ***P S S*** K ***R F*** Q ***P*** F^{548}	
	L T G T P P R S L P V L	3 (1, 2)
	A N E G A **P S S**– **R F** H **P**	4 (1, 2)
S protein	^{737}Q ***Y G*** S ***F*** C ***T Q*** L N ***R*** A L^{749}	
	S **YG** D **F** – **T Q** F T **R** H T	3 (1, 2)
	K **YG** L **F** S **T Q** P E A E	1 (2)
S protein	^{786}***Q I L*** P **D** P ***L*** K ***P*** T K R^{797}	
	Q I L H **D** D **L** P **P** H R V	1 (2)
S protein	^{1060}A P A I C H E ***G*** K A Y F ***P R*** E G **V** F V^{1078}	
	^{a}S D **G** Y D H P **P R** T R **V**	4 (1, 2)
	G **P** R L T **H** S **G** D L H L	2 (1)
S protein	^{1078}V F N ***G*** T S ***W F*** I T Q ***R*** N^{1090}	
	N L S **G** S T **W F** F SW **R**	2 (1, 2)
Group 2		
M protein	^{149}R M ***A*** G ***H*** S ***L*** G ***R*** C D I K D L P^{163}	
	T L **A** A H V **L** D **R** S P Y	1 (1)
	A S **H** P **L** G **R** C L S H T	1 (2)
M protein	^{173}P K E I T V ***A T S R T L*** S^{185}	
	S H V P L **A T S R T L** A	5 (1, 2)
	N H F V P Q P P **S R T** W	1 (2)
Group 3		
Without homology	T S N L G K A H T Q K L	1 (2)

Note. Each screen consisted of four rounds of panning with affinity-purified target antibodies. The numbers on either side of the sequence denote amino acid sequence numbers. Letters in bold denote matches of phage sequences with the *SARS-Cov* sequence and italicized bold letters denote conservative substitutions. The numbers on the right indicate the number of times the corresponding peptide was isolated and the numbers in parenthesis refer to the screen at which the peptide was isolated, i.e., the first screen, the second screen or both.

[a] The corresponding phage clone was chosen for competitive-inhibition assay.

the ratio of $CD4^+$ T cell/$CD8^+$ T cell was a little higher. However, the increase in $CD4^+$ T cell/$CD8^+$ T cell ratio was similar in the control group and all MAP-immunized groups, except the MAP2-immunized group on day 14. It suggested that these epitope-based peptides did not imbalance the immunological system. Subsequently, with the activation of T cells, the T cell/B cell ratio progressively returned to a normal level in all groups except the MAP2-immunized group at a low level. The results demonstrated that humoral immune response was predominant and was rapidly triggered by the epitope-based peptide, especially by MAP2.

Since the expression of surface molecules, CD44 and L-selectin, on $CD4^+$ T cells was used to distinguish memory cells (^{high}CD44, and ^{low}L-selectin), we monitored these phenotype changes using three-color flow cytometric analysis. The $CD4^+$ T lymphocytes of epitope-based peptide-immunized mice (day 0, just before the third immunization) showed the classical immunological memory phenotype (^{high}CD44, and ^{low}L-selectin) (Fig. 6). With the re-injection of the specific peptide, CD62L was continuously up-regulated (but its level was always lower than that of the PBS control). Slight down-regulation of CD44 on $CD4^+$ T cells stimulated by MAP1 and MAP4 on day 1 was observed; and then the level of CD44 returned to that on day 0, but subsequently fluctuated and remained higher than that of the PBS control. CD44 was continuously up-regulated on $CD4^+$ T cells from MAP2 and MAP3 challenged mice.

The frequency of IL-4- or IFN-γ-producing cells at the single cell level was determined by ELISPOT assay. As shown in Fig. 7, 7 days after primary immunization with MAP2 and MAP4, the number of IL-4-producing cells from spleens increased significantly (36–88 spots in 2×10^5 cells). Fourteen days after primary immunization with MAP1 and MAP3, the number of IL-4-producing cells remained elevated (31–43 spots in 2×10^5 cells). Moreover, the number of IL-4-producing cells in mice boosted with any of the peptides increased 2- to 3-fold, whereas the number of IFN-γ-producing cells remained at low levels (10–20 spots in 2×10^5 cells). These data demonstrate that the four B cell epitopes were more effective enhancers of humoral immunity than cellular immunity. The results of antigen-specific lymphocyte cell proliferation assay indicated that each MAP dose-dependently stimulated a proliferative response and MAP3 was the most effective inducer of lymphocyte proliferation (Table 3). Splenic lymphocytes from mice on day 42 still exhibited significant proliferative responses to specific antigen, demonstrating that the four epitope-based peptides induced long-term immune responses (data not shown).

Discussion

Phage-displayed peptide libraries have a wide variety of uses (Cortese et al., 1995; Petersen et al., 1995; Pereboeva et al.,

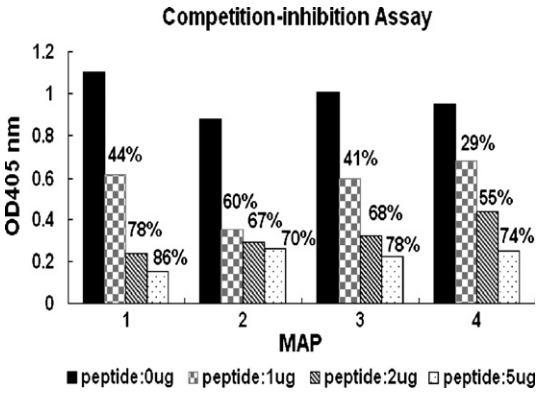

Fig. 2. Competitive ELISA was performed using different doses of MAPs (0–5 μg) as competitors of their corresponding phage clones. The percentage of inhibition is also shown.

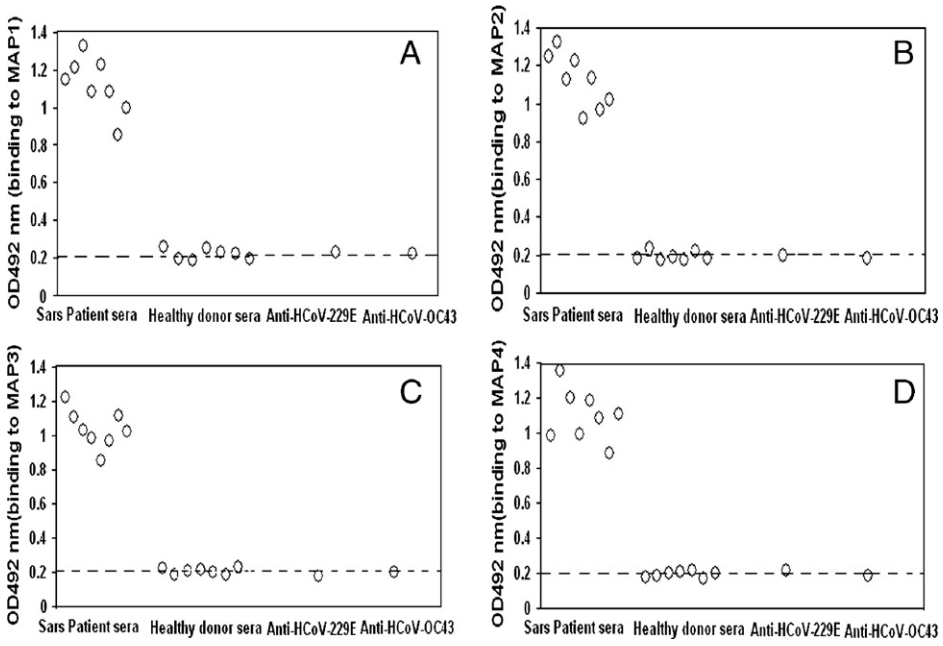

Fig. 3. Detection of the specificity of the epitope-based peptides reactive with convalescent sera: (A) MAP1, (B) MAP2, (C) MAP3, and (D) MAP4. Sera (1:100) were from 8 SARS patients, 7 healthy donors, and mice immunized with HCoV-229E and HCoV-OC43.The dashed line denotes the OD value of negative control.

1998; Wu et al., 2001). This technique has been used mostly to screen with monoclonal antibodies, and little to screen with purified polyclonal antibody preparations (Folgori et al., 1994; Kay et al., 1993; Roberts et al., 1993; Liu et al., 2004). We were successful in screening with polyclonal antisera using this technique, probably because of the newly improved biopanning we adopted and the targets we chose. Anti-*SARS-CoV* serum contains mostly *SARS-CoV* antibodies and few antibodies to other pathogens. In conventional biopanning processes with anti-*SARS-CoV* sera as the only targets, nonspecific phage clones that do not bind to *SARS-CoV*-specific immunoglobulins but instead to antibodies specific to other pathogens might be amplified. In addition, some researchers reported that non-specific clones not binding to the target but to the plastic of plate could also be chosen in several conventional biopanning procedures (Adey et al., 1995). We improved the biopanning procedure to avoid these possibilities and ensure specificity of selection. Anti-*SARS-CoV* IgG Fab (as the simplest fragments with specificity of antibodies) facilitated the antigen specificity of our biopanning procedure.

By repeating the four-round biopanning, we were able to select eleven *SARS-CoV* epitopes. They formed a subset of the linear epitopes. Moreover, we found another peptide sequence displayed by an immunopositive phage clone without homology to any primary sequence of *SARS-CoV* proteins. The structure of epitopes recognized and bound by B cell receptor (BCR) was shown to be linear or spatial by the classical experiments of Michael Sela 30 years ago (Sela, 1969). Phage-displayed peptide libraries are generated by shotgun cloning of random oligonucleotides into the 5′ ends of either the pIII or pVIII genes of filamentous phage. The peptides encoded by inserted nucleotides are displayed on the phage surface and have independent spatial structure (Cwirla et al., 1990; Felici et

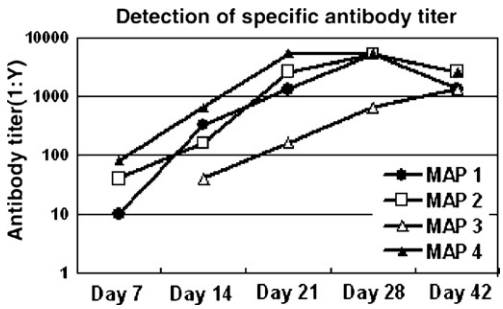

Fig. 4. Antibody response induced by MAPs in BALB/c mice. ELISA titers were calculated as the highest dilution resulting in a reading of 2 SD above the OD value of a negative-control serum and shown as mean titer+SEM.

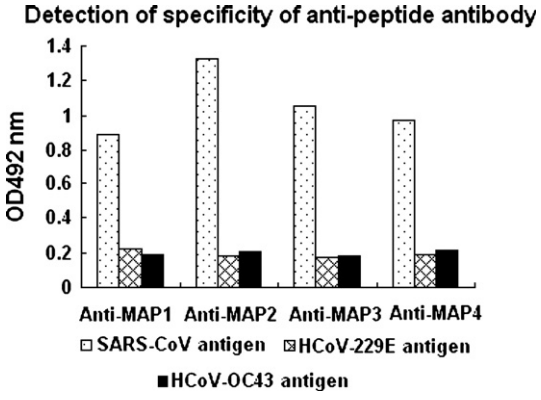

Fig. 5. Detection of the specificity of the anti-peptide antibody to SARS-CoV. In an ELISA, anti-peptide antibody from the immunized BALB/c mice reacted significantly with the inactivated SARS-CoV antigen but not with HCoV-229E or HCoV-OC43 antigen.

Table 2
The change in percentage of lymphocyte subsets during immunization of the four B cell epitope peptides of *SARS-Cov*

Groups	T, B cell subsets (% or ratio)					
	$CD3^+$(T)	$CD3^-CD19^+$(B)	T/B	$CD3^+CD4^+$(T4)	$CD3^+CD8^+$(T8)	T4/ T8
PBS control	41.88±0.39	51.90±1.31	0.81±0.02	26.18±1.86	13.60±1.68	1.94±0.25
FA control	40. 12± 0.31	50.73±0.98	0.80±0.01	24.22±1.33	12.75±1.62	1.96±0.28
MAP 1						
14 days	34.16±0.63[a]	55.76±0.95[a]	0.61±0.01[a]	22.43±1.49[b]	10.68±0.77[a]	2.19±0.16
21 days	33.58±0.64[a]	57.58±2.19[a]	0.58±0.02[a]	23.23±0.67[b]	11.56±0.85	1.95±0.24
35 days	43.08±0.71	52.88±0.96	0.814±0.01	25.8±0.59	13.30±1.45	1.96±0.25
MAP 2						
14 days	32.28±1.27[a]	58.90±1.66[a]	0.55±0.03[a]	22.44±0.82[b]	9.6±0.97[b]	2.35±0.21
21 days	34.76±1.21[a]	58.02±2.48[a]	0.60±0.04[a]	22.34±1.03[b]	10.66±0.72[b]	2.10±0.12
35 days	35.2 ±1.43[a]	54.4±1.91	0.64±0.03[a]	20.04±1.39[b]	8.83±0.76[a]	2.28±0.14
MAP 3						
14 days	22.74±0.26[a]	43.44±0.67[a]	0.52±0.01[a]	14.84±1.41[a]	7.22±0.36[a]	2.09±0.18
21 days	35.22±0.78[a]	58.14 ±1.17[a]	0.61±0.01[a]	22.20±1.64[b]	11.40±0.66[b]	1.94±0.06
35 days	40.84±0.71	50.56±0.90	0.81±0.00	24.34±1.98	11.74±0.40	2.07± 0.11
MAP 4						
14 days	25.60±0.51[a]	56.88±1.85[b]	0.50±0.01[a]	18.32±1.26[a]	8.82±0.95[a]	2.09±0.25
21 days	34.14±1.75[a]	52.70±1.85	0.65±0.02[a]	22.90±1.17[b]	13.00±0.70	1.772±0.15
35 days	38.14±0.96[a]	49.50±3.24	0.77±0.03	22.86±1.17[a]	13.68±0.67	1.68±0.14

Lymphocytes were from the mice immunized with the four different peptides on 14 days, 21 days, and 35 days after primary immunization ($n=5$). Results are means±SD.

[a] P value < 0.01 compared with the control group.

[b] P value < 0.05 compared with the control group.

al., 1993; Luzzago et al., 1993). So the non-homologous peptide sequence might reflect the presence of a discontinuous epitope of *SARS-CoV*. In addition, *SARS-CoV* is antigenically cross-reactive with other viruses, so the non-homologous peptide sequence might be also an epitope of a different virus. Certainly, this finding must be studied further. Among the linear epitopes, nine (81%) were considered to be S protein epitopes (five epitopes in S1 protein and four in S2 protein) and two (18%), M protein epitopes. The decrement tendency of numbers of epitopes of S1 protein, S2 protein, and M protein connected with the location of these proteins in the virion. Most (60%) of the epitopes were located in regions where predicted epitopes were known to exist, and a minority (24%) of epitopes were located about 6−13 amino acids away from the predicted epitope regions (Yuxian et al., 2005; Yanbo et al., 2003, 2004). And two were located in regions where the virus binds to the host cell receptor (ACE-II) (Wong et al., 2004).

S protein is responsible for inducing host immune responses and virus neutralization by antibodies (Holmes, 2003; Navas-Martin and Weiss, 2003), and neutralizing antibodies can protect mice or primates from *SARS-CoV* infection (Bisht et al., 2004; Buchholz et al., 2004; Bukreyev et al., 2004; Gao et al., 2003;Yang et al., 2004). Our research provided an indirect proof that the S protein of *SARS-CoV* is an important surface antigen stimulus of the host immune response. The M proteins of other coronaviruses are immunogenic (Enjuanes et al., 1995).The virion structure of *SARS-CoV*, B cell epitope characteristics, and our B cell epitope results suggest that M protein of *SARS-CoV* also plays an important role in inducing antibody production.

We sought to characterize the antigenicity and immunogenicity of the *SARS-CoV* epitope-based peptides to facilitate the development of effective SARS diagnostics and vaccines. Recent studies have demonstrated that *SARS-CoV* and other coronaviruses are antigenically cross-reactive (Ksiazek et al., 2003; Woo et al., 2004). The four S protein epitope-based peptides were proven *SARS-CoV* specificity, and antibodies from mice induced by them also reacted specifically with *SARS-CoV*. Though further study of their specificity and sensitivity is needed, these epitopes should be considered potential immunoreactive epitopes for diagnostic reagents.

The immunological effect of epitope-based peptides was studied. The lymphocyte proliferation assay showed the four epitope-based peptides stimulated the immune system. Secretion of IL-4 (a B cell growth and differentiation factor) might reflect the state of activation and proliferation of B cells and transition to antibody-producing plasma cells. The increase was dramatically greater in the number of IL-4-producing cells than in the number of IFN-γ-producing cells after immunization with these epitope-based peptides, confirming that humoral immunity was predominant. So, the epitopes we selected are real B cell epitopes. The specific antibody induced by the four epitopes persisted at least 6 weeks, which was confirmed by monitoring IgG titer. Although specific IgG could be detected after primary immunization, persistent high titer IgG required revaccination. It indicated the involvement of humoral immune memory. Within 1 week after revaccination, B cell numbers peaked, which indirectly proved that antigen-specific memory B cells could be induced by these epitope peptides. It suggested that

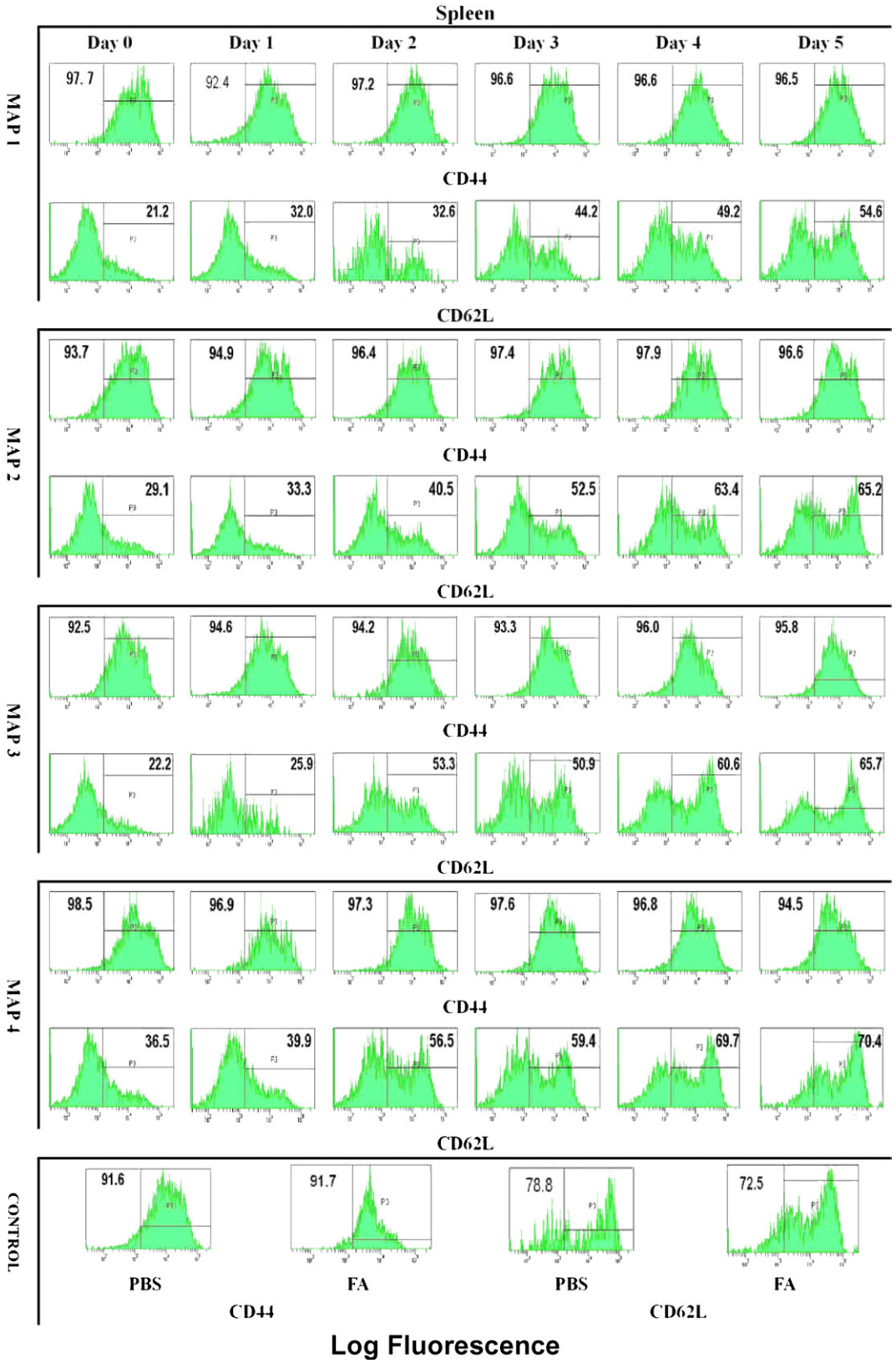

Fig. 6. Three-color flow cytometric analysis of surface markers (CD44 and CD62L) expression on $CD4^+$ T cells isolated from the spleens of immune mice before (on day 0) and after (on days 1, 2, 3, 4, and 5) the third immunization with MAP1–4. Gates were set on the $CD4^+$ T cells. The experiment was repeated three times with the same overall results. The values within each box indicate the relative cell number.

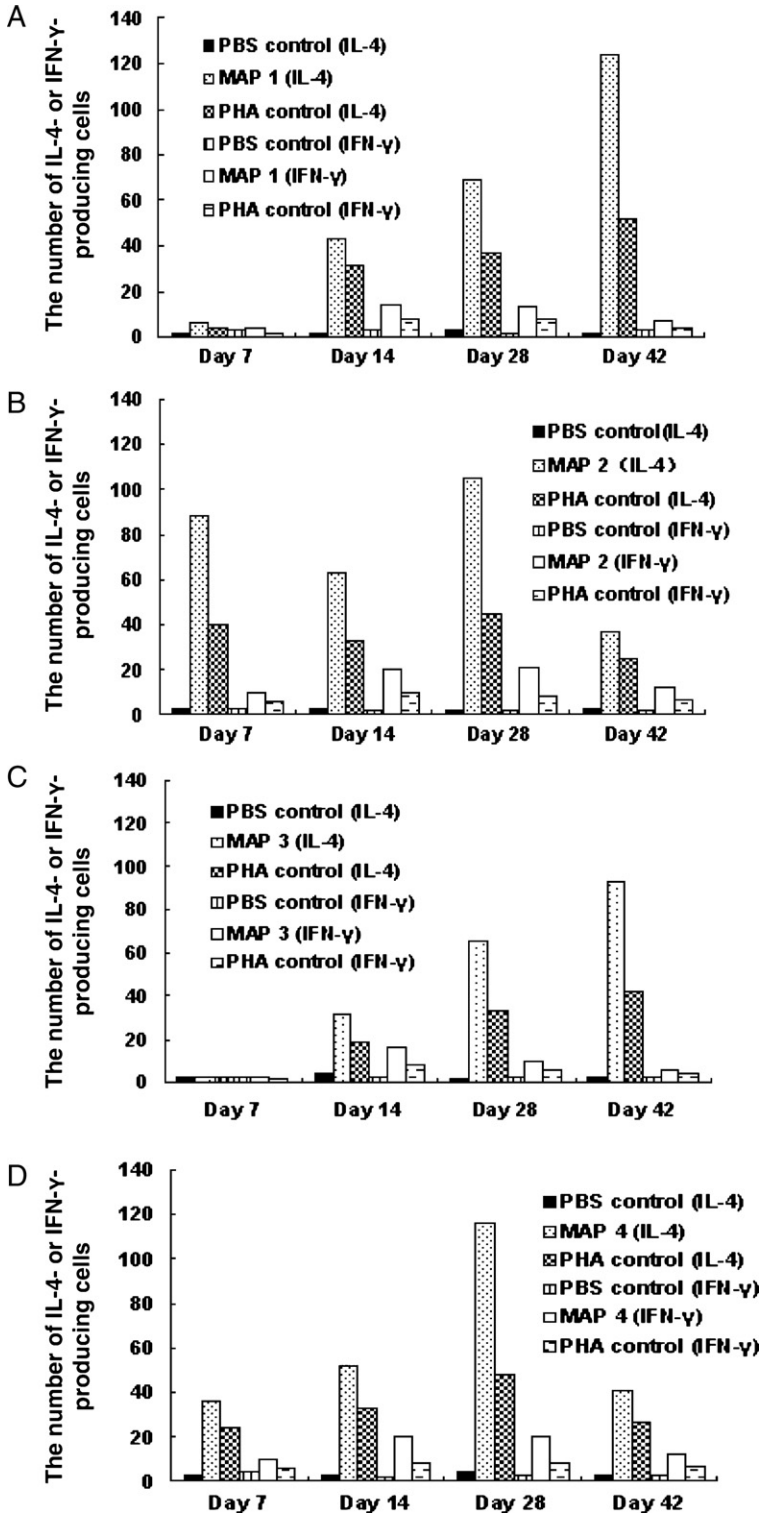

Fig. 7. The number of spots representing IL-4 or IFN-γ-producing cells was counted automatically with an ELISPOT reader. Splenocytes were harvested from mice challenged with four epitope-based peptides (MAP1, MAP2, MAP3, and MAP4) on 7 days, 14 days, 28 days, and 42 days after primary immunization. To determine the number of peptide-specific IL-4-producing cells and IFN-γ-producing cells in 2×10^5 lymphocytes, splenocytes were incubated with 5 μg ml^{-1} of the corresponding MAP for 20 h and 48 h respectively. (A) The number of MAP1-specific IL-4 or IFN-γ-producing cells. (B) The number of MAP2-specific IL-4 or IFN-γ-producing cells. (C) The number of MAP3-specific IL-4 or IFN-γ-producing cells. (D) The number of MAP4-specific IL-4 or IFN-γ-producing cells. PBS and PHA (5 μg ml^{-1}) were used as negative and positive control respectively.

Table 3
Lymphocyte proliferation response induced by epitope-based peptides

Immunization[a]	T cell stimulation index with stimulant[b]			
	MAPs (μg ml^{-1})			PHA (μg ml^{-1})
	2.5	5	10	5
MAP 1	18.4±2.6	36.1±2.0	23.1±1.9	31.2±2.8
MAP 2	14.3±2.2	17.6±2.1	28.1±2.4	36.7±2.3
MAP 3	22.7±2.6	41.8±1.7	47.6±2.9	36.4±1.9
MAP 4	16.1±1.3	19.8±1.3	37.2±3.2	40.6±1.3
PBS control	0.3±0.1	0.4±0.1	0.6±0.1	8.5±1.2
FA control	0.8±0.3	1.9±0.4	3.0±0.3	12.5±0.8

[a] BAL B/c mice were injected twice with 100 μg of epitope-based peptides in the form of MAP constructs. Mice receiving 300 μl of PBS or CFA served as negative controls. On day 7 after final immunization, splenocytes for the lymphocyte proliferation assay were pooled from immunized mice.
[b] Values are from one representative experiment out of five performed and are presented as the mean stimulation index (of quadruplicate wells)±SD.

these epitopes were potential for development of SARS vaccine. MAP2 and MAP4 induced rapid increase in the number of IL-4-producing cells to enhance the humoral immune indicated their potential uses for emergent prophylaxis of SARS; MAP1 and MAP3 induced relatively slow but persistent increase in that to enhance humoral immune indicated their potential uses for long-term prophylaxis of SARS.

Since activated Th ($CD4^+$ T) cells are also needed for effective humoral immunity, Th cells will be activated rapidly with the help of immune memory. In the mice, CD62L (Bradley et al., 1992; Yin et al., 1991) and CD44 (Budd et al., 1987; Butterfield et al., 1989) are used to distinguish naive from memory cells, and naive from activated T cells (Gerberick et al., 1997). In our study, in epitope-based peptide-immunized mice, the classical phenotype of immunologic memory ($CD44^{high}$, and L-$selectin^{low}$) was present on the surface of $CD4^+$ T lymphocytes just before the third immunization (day 0), suggesting that these epitope-based peptides could enhance the $CD4^+$ T memory cell response and thereby increase immune surveillance, defense, and capacity to carry out their auxiliary functions when specific antigen reappeared.

Because excessive or depressed immune responses can result in immunologic derangement and adversely affect the body, it is important to know whether epitope can keep steady state of the immune system especial for its using as constituent of vaccine. So we monitored the change in lymphocyte subsets numbers of splenic lymphocytes. Happily, the safety of the four B cell epitopes was confirmed.

Our study provided several *SARS-CoV* epitopes along with their immunological characteristics. These four epitopes had the ability to induce rapid, strong, and long-term humoral immunity, and they were also safe and effective at least in mice. Therefore, the four epitopes might have potential use as constituent of SARS vaccine. Moreover, our limited studies on antigenic specificity of the four epitopes suggested their potential use as diagnostic biomarkers. In addition, S and M proteins were important surface antigens of *SARS-CoV* confirmed by our results of immunoselection of B cell epitopes.

Materials and methods

Samples

Anti-*SARS-CoV* serum samples were collected from 8 convalescent-phase SARS patients (the diagnostic criteria for *SARS-CoV* infection followed the clinical description of SARS released by the World Health Organization) within a month after recuperation, tested positive for *SARS-CoV* antibody using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Huada Company, Peking, China). Antibody titers varied from 1:320 to 1:1280. Normal human serum samples (*SARS-CoV* seronegative) were pooled from seven blood donors. The *229E* and *OC43* standard strains of human coronaviruses (*HCoV-229E*, *HCoV-OC43*) and their specific antisera of murine origin were obtained from the Guangdong Center for Disease Control (CDC).

Synthetic polypeptides

Four epitope-based dodecapeptides were synthesized in the form of an eight-branch multiple antigen peptide (MAP) construct (purity > 85%; HuaCheng Company, Xi'an, China) and the coincidence of the actual molecular mass with the theoretical molecular mass was confirmed by mass spectrography analysis.

Animals

Female 6- to 7-week-old BALB/c mice were purchased from the Centre of Experimental Animals Sun Yat-sen University, Guangzhou, China. The Animal Experiment Ethics Committee of Sun Yat-sen University approved the protocols of all experiments performed in this study. All animals were handled according to the guidelines of the Chinese Council on Animal Care.

Immunoselection of B cell epitopes

Preparation of the targets

Immunoglobulin G (IgG) from convalescent and normal human serum was purified respectively using an affinity column, protein A Sepharose CL-4B (DingGuo Company, Peking China). Fab fragment of IgG (IgG Fab) was prepared by digestion of intact IgG with caroid, and then the digest was rechromatographed using another protein A Sepharose CL-4B column. The purity of the targets was checked by SDS-PAGE and their antigenicity was determined using ELISA and Western blotting. Human IgG and IgG Fab (Sigma Chemical, St. Louis, MO, USA) were used as references, and horseradish peroxidase (HRP)-labeled goat anti-human IgG and IgG Fab (Sigma) were used as detector antibodies in these identifications. The binding activity to *SARS-CoV* antigen of the target molecules was determined using ELISA.

Affinity selection using the phage-displayed peptide library

To enrich phage clones specific for anti-*SARS-CoV* IgG Fab, we improved the conventional biopanning procedure. The first round of biopanning was performed on immobilized anti-*SARS-*

CoV IgG Fab to ensure all phage clones binding to it were included in the pool of the first screen. In other words, 4×10^{10} phages of the original library (New England Biolabs, Ipswich, MA, USA) were immunoscreened with anti-*SARS-CoV* IgG Fab for 1 h at room temperature. Unbound phages were removed by washing and the bound phages were eluted with glycine–HCl (0.2 M, pH 2.2, and 1 mg ml^{-1} BSA) and neutralized with Tris–HCl (1 M, pH 9.0). After titration on LB/IPTG/X-gal (Promega, Madison, WI, USA) plates and amplification, 4×10^{10} first-round selected phages were immunoscreened with normal IgG Fab and the unbound phages were collected to remove phages not specific for anti-*SARS-CoV* IgG Fab in the second biopanning. The protocols for the third and fourth immunoscreen were identical to that of the first round except 2×10^{11} phages particles were used as input and the Tween 20 concentration was increased for washing to 0.5% (v/v). The four rounds of biopanning were repeated once.

Identification of target phage clones

Thirty blue plaques from each fourth-round titration plate were picked at random, amplified in host bacteria (*E. coli* ER2738), and precipitated with polyethylene glycol (PEG)/NaCl. ELISA was performed on 96-well plates coated respectively with 10 μg ml^{-1} anti-*SARS-CoV* serum mixture and normal human serum mixture. After blocking, 10^{12} phage particles of each clone per well were incubated for 2 h. Then, the plates were washed at least six times with TBST (0.5%). HRP-conjugated anti-M13 antibody (Pharmacia, Biotech, Piscataway, NJ, USA), diluted in BSA blocking buffer (1:5000), was added to the plates and incubated for 1 h. After washing and reacting with substrate 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) for color development for 20 min in the dark, the absorbance at 405 nm was measured in a Microplate reader (Multiskan MK3, Thermo Labsystems, Shanghai, China). The equal amount of wild type phages was used as a negative control simultaneously. Immunopositive phage clones were preserved as target clones for further study.

DNA sequencing and homology analysis

Single-stranded DNA (prepared from each target clone as template) and the −96 gIII primer were used for sequencing. Sequences of DNA inserted into target phage clones were translated into amino acid sequences and compared with that of structural proteins of *SARS-CoV* (AAS48453, AAS48454, AAS48455, AAS48456, AAU07933, AAT74874, etc.) which were retrieved from the Genebank site (www.NCBI.NLM.NIH.GOV/) using Standard protein–protein BLAST and Clustal W Multiple Sequence Alignment public software. We analyzed several properties of these epitopes including hydrophilicity, antigenicity, accessibility, flexibility, polarity, and secondary structure and chose four predominant epitopes (dodecapeptides) to synthesize for further study.

Properties analysis of synthetic peptides

Competitive-inhibition assay. In competitive-inhibition experiments, coating with anti-*SARS-CoV* serum, blocking, and washing were performed as above. Different amounts of the four synthetic peptides and their corresponding phage clones were added simultaneously and incubated for 1 h at 37 °C. Then the same steps as above were followed and absorbance was measured. The inhibition percentage was calculated as follows: inhibition% = [(OD_{405} without competitor − OD_{405} with competitor)/OD_{405} without competitor] × 100%.

Binding assay. To assess the specificity of the epitope-based peptides, their ability to bind to normal human, anti-*SARS-CoV*, anti-*HCoV-299E*, and anti-*HCoV-OC43* sera were examined by ELISA. Each of the above antisera was incubated (1 h, 37 °C) in ELISA wells coated with 10 μg ml^{-1} epitope-based peptides. After being washed, the wells were incubated with corresponding HRP-conjugated second antibody for 1 h, then *o*-phenylenediamine (OPD) to develop the color, and finally, optical density (OD) values at 492 nm were measured.

Evaluation of immunological effect induced by epitope-based peptides

Immunization

For monitoring specific antibody titer, cytokine levels, and lymphocyte proliferation, mice were randomly divided into six groups (one group for each of the four peptides, one for the PBS control, and one for the Freund adjuvant (FA) control, $n=5$). Mice were immunized subcutaneously with 100 μg of peptide as immunogen in the presence of Freund complete adjuvant and boosted with freshly prepared emulsion of the immunogen and Freund incomplete adjuvant at 2-week intervals. Generation of immunological memory in mice was by a second immunization and was followed by an 8-week rest period. For the study of the $CD4^+$ T cell recall response, mice with immunological memory were immunized with 100 μg of peptide without adjuvant (Akbar et al., 1993) for a third time.

IgG ELISA for specific antibody

Blood samples were obtained from the lateral tail vein of mice on days 7, 14, 21, 28, and 42 after primary immunization. Antibody titer was measured using synthetic peptides as coating antigens for an IgG ELISA. *HCoV-229E* and *HCoV-OC43* viral suspension with a titer of 5×10^6 $TCID_{50}$ ml^{-1} was used to prepare the virus antigen. Antibody specificity was determined from the IgG ELISA reactivity of sera with the antigens, *SARS-CoV*, *HCoV-229E*, and *HCoV-OC43*, respectively.

Purification of splenic lymphocytes

Spleen was cut into pieces and carefully forced through a metal mesh. For flow cytometric analysis, splenocyte suspensions were treated with 1× RBC lysis buffer (eBioscience, San Diego, CA, USA) to lyse erythrocytes, then washed and suspended up to concentration of 2×10^7 cells ml^{-1} in staining buffer (D-PBS pH 7.0/0.2% NaN_3/5% calf serum). For the enzyme-linked ImmunoSPOT (ELISPOT) and lymphocyte proliferation assays, lymphocytes were separated from the splenocyte suspensions by density gradient centrifugation and

suspended (2×10^6 cells ml^{-1} in RPMI 1640). All lymphocyte samples were prepared and used freshly.

Flow cytometric analysis

Fifty microliters of suspended lymphocytes prepared as above, which were harvested, respectively, from twice-immunized mice on days 14, 21, and 35 after the first immunization and from boosted memory mice on days 0, 1, 2, 3, 4, and 5 after the third immunization, were incubated with a panel of anti-mouse antibodies against the surface molecules fluorescein isothiocyanate (FITC)-conjugated CD3e, phycoerythrin (PE)-conjugated CD19, CD4, CD8a, allophycocyanin (APC)-conjugated CD44, and CD62L (L-selectin, eBioscience). After incubation at 4 °C for 20 min in the dark, cells were washed three times before being suspended in 4% paraformaldehyde and analyzed using a BD FACS Aria™ instrument (Becton Dickinson, Franklin Lakes, NJ, USA). Antibody-isotyping control flow cytometric analyses were carried out essentially in the same manner. The data were analyzed using BD FACSDiva software.

Assessment of peptide-specific IL-4- or IFN-γ-producing cells using the ELISPOT technique

IL-4- or IFN-γ-producing cells were assessed by specific ELISPOT kit according to the manufacturer's protocol (U-Cytech, Utrecht, The Netherlands). Briefly, 100 μl of lymphocytes from immunized mice on days 7, 14, 28, and 42 after primary immunization were incubated with corresponding peptides or PHA at a final concentration of 5 μg ml^{-1} in wells coated with captured interleukin-4 (IL-4) antibody or interferon-γ (IFN-γ) antibody after the wells were washed and blocked. After proper incubation at 37 °C with 5% CO_2, the cells were removed by washing, and the secreted cytokine was detected with biotin-labeled anti-murine IL-4 or IFN-γ monoclonal antibody. The spots were counted with an automatic reader.

Lymphocyte proliferation assay

One hundred microliters of prepared lymphocytes was cultured in quadruplicate in 96-well plates in the presence of 100 μl of the corresponding peptides at final concentration of 2.5 μg, 5 μg, and 10 μg ml^{-1}, respectively, and the control PHA was at 10 μg ml^{-1}. After incubation (37 °C, 68 h), 100 μl of supernatant was removed and 10 μl of 3-(4, 5 dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT, 50 μg ml^{-1}) was added. After further incubation for 4 h, the lymphocytes were lysed (100 μl of dimethylsulfoxide [DMSO], room temperature for 20 min) for developing color and the OD values at 570 nm were measured.

Statistical analysis

Student *t* test was used for statistical analysis.

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