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## The development of Chinese specific human cytomegalovirus polyepitope recombinant vaccine

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### ABSTRACT

Human cytomegalovirus (HCMV) infection is a major cause of morbidity in the recipients of organ transplants and in the congenitally infected infants. HCMV vaccine has emerged as an effective approach to prevent HCMV infection particularly for the development of multiple viral antigens vaccination and human leukocyte antigen (HLA)-restricted polyepitope technology. As the Chinese population makes up more than one fifth of the population worldwide, it is important to develop HCMV vaccines more specific for the Chinese population by targeting Chinese-restricted HLA alleles and antigens. In the present study, we designed a novel chimeric polyepitope vaccine based on the replication-deficient adenovirus Ad5F35, which encodes 83 HCMV T cell epitopes from 15 different HCMV antigens, restricted to 14 HLA I and 7 HLA II alleles that cover 92% of the Chinese population. Our results show that the recombinant adenovirus vaccine Ad5F35-CTL.Th can be efficiently transfected and expressed in peripheral blood mononuclear cells (PBMCs) with little cytopathic activity. Ad5F35-CTL.Th can also be endogenously processed and presented by PBMCs. Ad5F35-CTL.Th-stimulated HCMV-specific cytotoxic T lymphocytes (CTLs) showed strong cytolytic activity against HCMV polyepitope-sensitized target cells. The CTL activity was accompanied by high levels of IFN- $\gamma$  production after Ad5F35-CTL.Th stimulation. The specificity and vigorous response to the recombinant adenovirus vaccine in vitro makes it a potential candidate to be used for transplantation recipients or congenitally infected infants.

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### 1. Introduction

Human cytomegalovirus (HCMV) is found universally throughout all geographic locations and socioeconomic groups, with seroprevalence ranging from 45% to 100% (Cannon et al., 2010). HCMV can cause clinical disease in the fetus, neonate and immunocompromised individuals such as transplant recipients and HIV-infected patients. Congenital HCMV infection is an important cause of hearing, cognitive, and motor impairments in newborns (Dollard et al., 2007; Hyde et al., 2010). In the allograft recipient, viremic dissemination can cause end-organ disease, such as hepatitis, pneumonitis, gastroenteritis and retinitis, and can predispose for transplant rejection. Developing a vaccine in the two populations

has been rated as high priority. Recently, two HCMV phase 2 clinical trials have now been completed with positive results from the induction of humoral immunity, which showed that HCMV glycoprotein B (gB) vaccine has the potential to decrease incident cases of maternal and congenital CMV infection (Griffiths et al., 2011; Pass et al., 2009). We have also generated vaccine Ad5F35-AD-1 that expressed the conserved conformational epitope AD-1 from gB protein (Zhao et al., 2009). Moreover, cellular immunity provided by the CD8+ and CD4+ T lymphocytes has shown to play a key role in resolving the primary infection, providing long-term immune surveillance, and the clearance of replicating virus (Crough et al., 2007; Fornara et al., 2011; Nebbia et al., 2008). Therefore, it is of great importance to develop an effective HCMV vaccine based on viral antigens that activate cellular immunity to prevent HCMV-associated disease (Bunde et al., 2005; Einsele et al., 2002).

Over the past 30 years, various strategies of cellular immunity, including the use of subunit vaccines, its DNA or dense bodies to vaccinate, have been developed and promising results in preclinical studies have been demonstrated (Khanna and Diamond, 2006; Plotkin, 1999; Schleiss, 2005, 2007). However, the success

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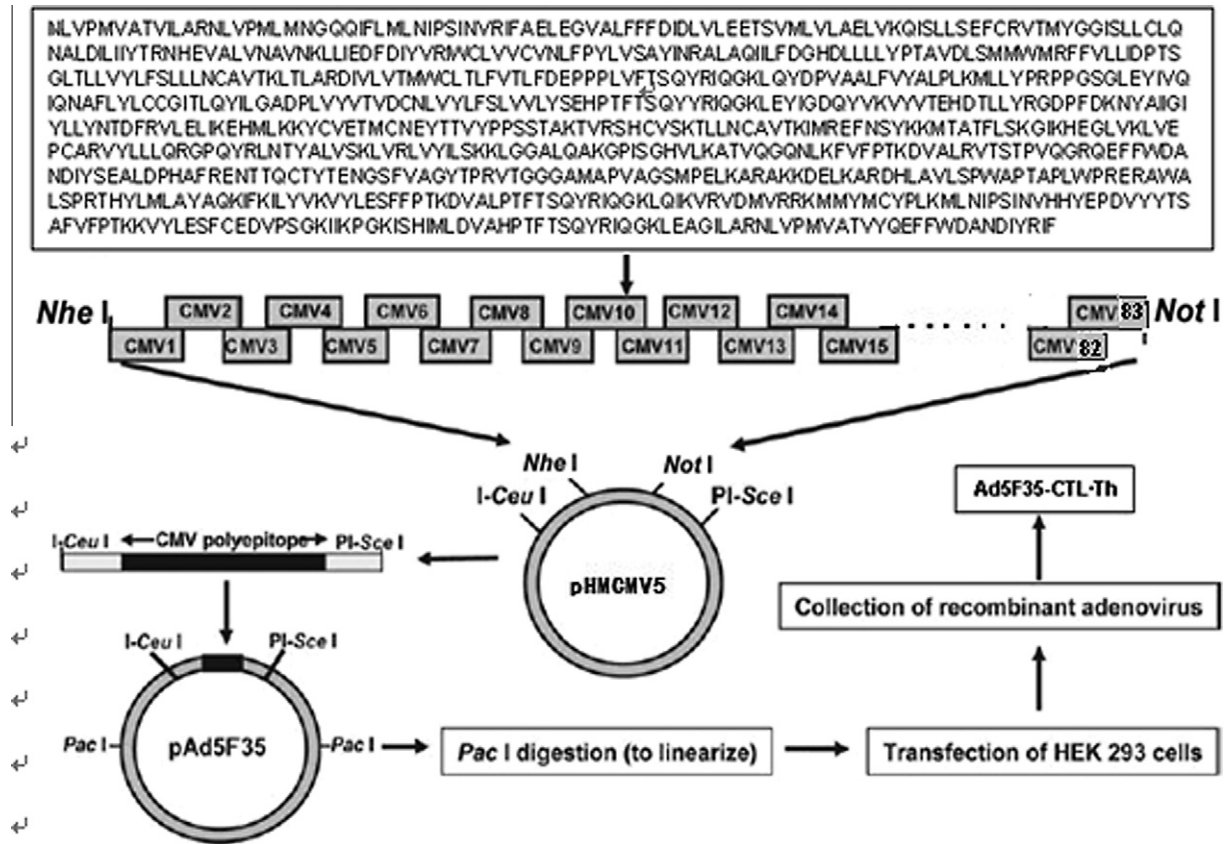
of the strategies is hindered by the limited range of epitopes and HLA alleles that could be included to develop the HCMV vaccine. Ideally, a formulation should cover most viral antigens, which could induce a broad repertoire of HCMV-specific cellular immune responses. Recently developed polyepitope technology allows the presentation of multiple HLA class I-restricted CTL (cytotoxic T lymphocyte) epitopes derived from different antigens within a single reading frame and thus provides a new platform to achieve this goal. The epitope-based vaccine should also contain a minimal number of epitopes which cover a vast majority of the human population (Buteau et al., 2002; Fischer et al., 2007; Khan et al., 2006). T-cell epitopes that bind to multiple alleles of HLA are prime targets for vaccine and immunotherapy development due to their relevance to a large proportion of the human population, for example, SARS coronavirus nucleocapsid (Gupta et al., 2006), HIV-1 proteins (Berzofsky et al., 1991; Brown et al., 2003; Surman et al., 2001), and *Chlamydia trachomatis* outer membrane protein (Kim and DeMars, 2001). Epitope-based vaccines can effectively induce cellular immunity by selecting a small number of hotspots that can elicit most of the required T-cell functions. To date, many studies have focused on HLA-restricted HCMV epitope vaccines and progress has been made (Elkington et al., 2003; Rist et al., 2005). However, these investigations were mainly aimed at the Caucasian population or for a general population with limited coverage of HLA alleles or antigens. Since Chinese population with more than 50 different ethnic groups makes up one fifth of the world population, it is essential to develop HCMV epitope vaccines more specific to the Chinese population by targeting on more Chinese-restricted HLA alleles and broader antigens.

To develop HCMV vaccines specific for Chinese population, we designed a novel chimeric polyepitope vaccine based on the replication-deficient adenovirus Ad5F35 and evaluated their immunological effects *in vitro*.

**2. Materials and methods**

*2.1. Prediction and selection of Chinese HLA-restricted HCMV polyepitope*

The prediction and selection of Chinese HLA-restricted HCMV polyepitope were performed according to our forecasting system previously published on spatial coverage of cumulative phenotypic frequency (CPF) of HLA-I (Xue et al., 2005a,b). The CPF algorithm allows the identification of alleles that represent a desired percentage of a population, and also allows prediction of theoretical responder status in any given population where HLA gene frequencies are known. Briefly, the CPF of 14 HLA allele sites (A2, A24, A1, A3, A11, A68, B44, B7, A23, A26, B35, B38, B8 and B27) in the Chinese population was forecasted and the immunoactivity of HLA-restricted HCMV polyepitope nucleotide vaccine in different geographical locations was evaluated. Utilizing literature data on HLA-restricted HCMV epitopes that have been published, we selected 76 CTL epitopes and 7 Th epitopes derived from different HCMV protein antigens that can induce cellular immunologic response and stably bind corresponding class I and class II HLA molecules. Through SYFPEITHY (Rammensee et al., 1999) combined with MAPPP algorithms (Hakenberg et al., 2003), the selected



**Fig. 1.** Schematic view of the construction of a recombinant adenovirus that expresses a synthetic DNA encoding for a polyepitope protein which contains 83 HCMV T-cell epitopes. The DNA sequence encoding this polyepitope protein was synthesized using overlapping epitope sequence specific primers as described in “Section 2”. This synthetic insert was cloned into a T plasmid prior to cloning into the pHCMV5 vector. After amplification in *E. coli*, the expression cassette from pHCMV5 was excised and ligated into the pAd5F35 expression vector. Following linearization of the DNA using Pac I restriction enzyme, the recombinant pAd5F35 vector was packaged into infectious adenovirus by transfecting HEK 293 cells, and recombinant adenovirus (referred to as Ad5F35-CTL-Th) was harvested from transfected cells by repeated freeze–thawing cycles.

epitopes and polyepitope were predicted for the specific HLA-binding value and proteasomal cleavage site.

## 2.2. Construction and validation of a recombinant adenovirus with Chinese HLA-restricted HCMV polyepitope

The design and construction of a recombinant adenovirus vaccine expressing CTL.Th polyepitope of HCMV is shown in Fig. 1. The amino acid sequence of the 83 HLA restricted CTL.Th epitopes was translated into the nucleotide sequence using human universal codon usage, and the polyepitope DNA sequence representing the 83 epitopes has been synthesized using Splicing by Overlap Extension and stepwise asymmetric Polymerase Chain Reaction (PCR) (Thomson et al., 1995). The final DNA sequence was coded with a Kozak sequence, a start codon, 83 contiguous minimal

HCMV CTL.Th epitopes (Table 1), and a hexahistidine tag. After being cloned into a T vector and sequenced, the CTL.Th sequence was inserted into shuttle plasmid pHMCMV5 (kindly provided by Hiroyuki Mizuguchi, Osaka University, Osaka, Japan). The expression cassette was then cloned into Ad5F35 vector (provided by Hiroyuki Mizuguchi) and verified by restriction digestion and sequencing. The recombinant Ad5F35 was linearized and transfected into human embryonic kidney (HEK) 293 cells for 7 days until maximal cytopathic effect (CPE) was observed, pAd5F35-GFP was included as a vector control. The recombinant adenoviruses (Ad5F35-CTL.Th) were harvested from the transfected cells by successive freeze–thawing cycles. The titer of the large-scale Ad5F35-CTL.Th virus preparation was measured by plaque assay.

To confirm the successful development of the recombinant adenovirus, PCR was used to amplify the DNA extracted from

**Table 1**  
CTL.Th epitopes scores of SYFPEITHI and MAPP.

Epitope order	HLA restriction	HCMV antigen	Amino acid position	Abbreviated code	SYFPEITHI score	MAPP score
1	A2	pp65	495–503	NLV	30	1.000
2	A2	pp65	491–500	ILA	24	0.510
3	A2	pp65	320–328	LMN	23	0.940
4	A2	pp65	120–128	MLN	22	0.846
5	A2	pp65	522–530	RIF	23	0.825
6	A2	pp65	347–355	ALF	23	0.502
7	A2	IE-1	316–324	VLE	24	0.538
8	A2	IE-1	81–89	VLA	27	
9	A2	IE-1	303–311	SLL	24	
10	A2	IE-1	297–305	TMY	27	
11	A2	IE-1	125–133	CLQ	24	0.501
12	A2	IE-2	412–420	IYI	24	0.999
13	A2	pp150	195–203	ALV	29	0.869
14	A2	pp150	271–279	LIE	21	0.538
15	A2	gB	4–12	RIW	26	0.532
16	A2	gB	784–792	NLF		
17	A2	gB	493–501	YIN	25	0.501
18	A2	gH	197–205	ILF	26	0.506
19	A2	gH	395–403	LLY	27	
20	A2	US2	200–209	SMM	21	0.744
21	A2	pp28	240–248	LLI	29	0.925
22	A2	US3	161–169	TLL	29	0.644
23	A2	pp50	230–238	LLN	29	0.999
24	A2	UL16	185–193	TLA	28	
25	A2	UL18	3–11	TMW	23	
26	A2	US11	24–32	TLF	23	0.504
27	A24	pp65	369–379	FTS	13	0.999
28	A24	pp65	341–349	QYD	24	
29	A24	pp65	113–121	VYA	22	
30	A24	UL16	161–169	LYP	22	
31	A24	pp71	299–308	EYI	22	
32	A24	US6	150–158	LYL	22	0.501
33	A24	IE-1	353–361	QYI	22	0.523
34	A24	US2	181–189	VYV	23	0.999
35	A24	US3	164–172	VYL	24	0.999
36	A1	pp65	363–373	YSE	29	0.500
37	A1/B27	pp65	373–381	YRI	27	
38	A1	pp65	219–227	IGD	28	0.500
39	A1	pp50	245–253	VTE	34	0.501
40	A1	pp50	274–253	RGD	26	
41	A1/A3	gH	729–737	AII	20/25	
42	A1	gB	657–665	NTD	21	0.657
43	A1	IE-1	100–108	IKE	24	0.709
44	A1	IE-1	279–287	CVE	24	0.500
45	A3	pp150	945–955	TTV	29	0.501
46	A3	pp50	52–60	TVR	29	0.837
47	A3	pp50	229–237	TLL	34	0.504
48	A3	gB	683–691	IMR	19	
49	A3	gB	370–378	KMT	20	
50	A3	pp28	212–220	GIK	29	
51	A3/A1	pp28	269–277	LVE	26	0.503
52	A3	pp65	355–363	LLL	26	0.528
53	A3	gH	122–130	RLN	30	

Table 1 (continued)

Epitope order	HLA restriction	HCMV antigen	Amino acid position	Abbreviated code	SYFPEITHI score	MAPPV score
54	A3	gH	420–428	LVR	29	
55	A3	IE-1	184–192	KLG	26	
56	A11	pp65	16–24	GPI	21	
57	A11	pp65	501–509	ATV	25	0.501
58	A68	pp65	186–196	VVF	17	0.501
59	A68	pp150	794–802	VTS	22	0.573
60	B44	pp65	512–521	QEF	22	0.519
61	B44	gH	31–39	SEA	26	
62	B44	gH	54–62	REN	22	
63	B44	UL18	125–133	TEN	22	
64	B7	pp65	417–426	TPR	19	0.502
65	B7	US11	12–20	APV	24	0.858
66	B7/B8	IE-1	192–200	KAR	14	
67	B7	pp150	101–109	KAR	15	
68	B7	pp150	624–632	SPW	27	0.631
69	B7/B8	pp150	641–649	WPR	33	
70	B7	gH	690–698	SPR	24	0.662
71	A23	IE-1	248–257	AYA		0.987
72	A26	pp65	223–231	YVK	23	0.563
73	B35	pp65	188–195	FPTL		
74	B38	pp65	367–379	PTF		
75	B8	IE-1	88–96	QIK	24	
76	B27	IE-1	201–209	RRK	23	1.000
77	DR1	pp65	117–131	PLK	28	
78	DR1/3	pp65	510–524	YQE	32	
79	DR4/7	pp65	281–295	IJK	18	
80	DR7	pp65	177–191	EPD	22	
81	DR11/13	pp65	366–380	HPT	26	
82	DR11	pp65	489–503	AGI	14	
83	DR15	pp65	225–239	KVY	24	

Ad5F35-CTL.Th virions. Reverse Transcription (RT)-PCR and Western blotting were used to confirm the mRNA and protein expression of CTL.Th in HEK293-CTL.Th cells, while HEK293-GFP and parental HEK293 cells served as negative controls.

### 2.3. *In vitro* stimulation of PBMCs by Ad5F35-CTL.Th adenovirus vaccine

PBMCs from healthy donors were cultured in 6-well plates, and then infected with Ad5F35-CTL.Th or Ad5F35-GFP for 6 h. On day 1, 2, 6, and 10 post-infection, CTL.Th expression was analyzed by immunocytochemistry as follows. Briefly, PBMCs placed on slides were fixed with 4% paraformaldehyde. Non-specific antibody sites were blocked with 10% goat serum, then the cells were incubated with anti-6×His tag antibody (Abcam, Cambridge, England) or relevant isotype control antibody at 4 °C overnight. Slides were washed with PBS and then incubated with HRP-conjugated secondary antibody. All slides were processed by the Streptavidin-Peroxidase (SP) method (Zhongshan Co. Ltd., Beijing, China) for 30 min. Following staining with diaminobenzidine, slides were visualized using an OLYMPUS-BX51 microscope to observe the expression and localization of recombinant polyepitope or its proteasomal cleaved epitopes. The viability of infected PBMCs was determined by trypan blue staining.

### 2.4. Establishment and maintenance of cell lines

Epstein Barr virus-transformed lymphoblastoid cell lines (LCLs) were established from HCMV-seropositive donors by exogenous virus transformation of peripheral B cells using the B95.8 virus isolates. Autologous LCLs, pulsed with HCMV peptides and Ad5F35-CTL.Th, were used to stimulate T cell lines and clones on a weekly basis or as target cells in cytotoxicity assays (see below). All cell lines except HEK293 were routinely maintained in RPMI1640 medium supplemented with 100 units/ml penicillin G sodium, 100 µg/ml streptomycin, 4 mmol/L L-glutamine and 10% FCS. The HEK293

cell line was maintained in DMEM containing 10% FCS. The HCMV experimental strain AD169 was cultured on fibroblasts.

### 2.5. Synthesis of peptides

Peptides, synthesized by the Merrifield solid phase method, were purchased from Shanghai Sangon Biological Engineering Company. They were dissolved in 10% dimethyl sulphoxide, and diluted in serum-free RPMI1640 medium. Purity of these peptides were examined by HPLC and proven to be >94%.

### 2.6. Subjects

Peripheral blood was collected from 10 donors. DNA was extracted to test HLA alleles by PCR-SSP (sequence-specific primers), and serum was used to detect HCMV status by ELISA. Informed consent was obtained from each donor, and ethical approval for the study was obtained from the Ethical Committee of Qilu Hospital, Shandong University.

### 2.7. Generation of polyclonal and clonal HCMV-specific CTLs using HCMV peptides

Polyclonal CTL lines specific for HCMV epitopes were established according to methods previously published (Elkington et al., 2003; Khanna, 1998). Briefly,  $2 \times 10^6$  PBMCs from healthy virus carriers were cocultured with autologous x-irradiated PBMCs ( $1 \times 10^6$ ) pulsed with 20 µmol/L peptide for 16 h. On day 3, growth medium with rIL-2 was added, and the cells were further expanded. On day 7, the cultures were restimulated with peptide-sensitized, x-irradiated autologous LCLs. After 10 days in culture medium, the cells were used as polyclonal effectors in an *in vitro* cytotoxicity assay against peptide-sensitized autologous LCLs.

### 2.8. Expansion of HCMV-specific CTLs using Ad5F35-CTL.Th

HCMV specific CTLs were generated from the healthy virus carriers by stimulating PBMCs with Ad5F35-CTL.Th. For adenovirus

polyepitope stimulation,  $2 \times 10^6$  PBMCs from healthy virus carriers were cocultured with autologous x-irradiated PBMCs ( $1 \times 10^6$ ) which were infected with Ad5F35-CTL.Th (MOI of 100:1, 50:1, or 25:1) at a responder to stimulator ratio of 2:1. On day 3, the cultures were supplemented with growth medium containing rIL-2. On day 7, the cultures were restimulated with adenovirus-infected (MOI 50:1), x-irradiated autologous LCLs and supplemented with growth medium containing rIL-2. HCMV-specific T-cell reactivity (stimulated once or twice) was sequentially assessed on day 14 using an *in vitro* cytotoxicity assays and IFN- $\gamma$  production.

### 2.9. Cytotoxicity assays

The LDH method was used to detect the cytotoxicity of HCMV-specific CTLs. Briefly, the effector cells (E) and the target cells (T) were seeded in a 96-well plate at different E:T ratios. After 4 h of incubation, 100  $\mu$ l cell-free supernatant was transferred to a new 96-well plate and 100  $\mu$ l reagent was added into each well. Thirty minutes later, the LDH release was detected by spectrophotometer at 490 nm. Maximal or spontaneous LDH release was obtained by adding 1% Triton X-100 or complete medium to the target cells, respectively. The percentage of specific lysis was calculated by the following formula: % specific lysis =  $100 \times (\text{sample release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$ .

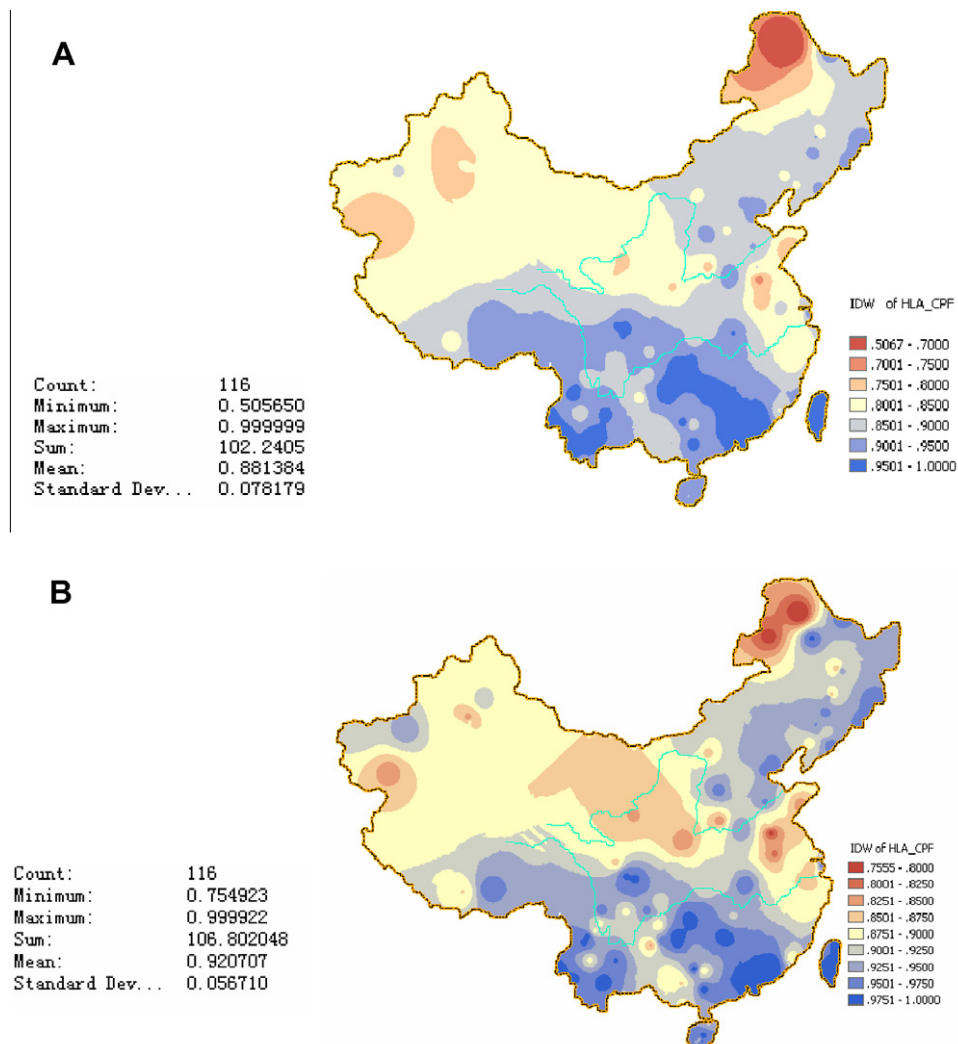
### 2.10. ELISPOT assay

PBMCs that were stimulated with Ad5F35-CTL.Th (MOI of 100:1, 50:1), or CTLs that were restimulated with Ad5F35-CTL.Th (MOI 50:1) were detected with ELISPOT to measure the production of IFN- $\gamma$  according to the protocol (R&D, Minneapolis, MN). Cytokine-producing cells were detected as purple spots, and the developed microplate was analyzed by counting spots using a specialized ELISPOT reader. Quantitation of results was done by calculating the number of spot forming cells (SFC) per number of cells added into the well. T cell precursor frequencies for each epitope were based on the total number of cells and the number of SFC per well (average of triplicate wells). Ad5F35-CTL.Th-specific spots were calculated after subtraction of the number of spots in negative control wells.

## 3. Results

### 3.1. Chinese HLA-specific HCMV polyepitope was successfully predicted and selected

Predominant Chinese-restricted alleles (A2, A24, A1, A3, and A11) were put into the algorithm of CPF, and the CPF of the set of alleles was 88.1% in the Chinese population (Fig. 2A). Then, other alleles (A68, B44, B7, A23, A26, B35, B38, B8, and B27) were added



**Fig. 2.** The CPF of the set of alleles in Chinese population. (A) The mean CPF of the set of predominant alleles (A2, A24, A1, A3, A11) was 88.1%. (B) The mean CPF of the set of alleles restricted to Chinese population (A2, A24, A1, A3, A11, A68, B44, B7, A23, A26, B35, B38, B8 and B27) was 92.1%.

to the algorithm, and the total CPF of these 14 HLA alleles reached 92.1% in Chinese population (Fig. 2B).

Based on the selected set of HLA alleles, we selected 76 CTL epitopes and 7 Th epitopes in different HCMV antigens that could induce wide-coverage immune response and stably bind class I and class II HLA molecules. Then the selected epitopes were put into the SYFPEITHI algorithm and the predicted scores of the selected epitopes has reached above 14, most between 20 and 30, indicating high HLA-binding affinity. MAPPP could predict both the proteasomal cleavage and peptide anchoring to HLA I molecules. The selected polyepitope sequence was put into the MAPPP algorithm. Most of the predicted proteasomal cleavage values reached a score above 0.5, suggesting the high possibility for single epitope production and presentation (Table 1).

The epitopes included in this polyepitope were derived from 15 different viral proteins (pp28, pp50, pp65, pp150, pp71, gH, gB, IE-1, IE-2, US2, US3, US6, US11, UL16 and UL18) that were involved in virus attachment, replication, assembly, reactivation and immune escape from the latent phase, all of which are crucial stages in the progression of HCMV infection.

### 3.2. Construction and characterization of the recombinant adenovirus vector pAd5F35-CTL.Th

The full-length CTL.Th nucleotide sequence has been synthesized, sequenced, and cloned into pHMCMV5. Double digestion of pHMCMV5-CTL.Th with *NheI/NotI* or *I-CeuI/Pi-SceI* produced expected fragments confirming the construction of pHMCMV5-CTL.Th. The 3602 bp CTL.Th expression cassette from pHMCMV5-CTL.Th was then cloned into the pAd5F35 vector to produce pAd5F35-CTL.Th. Double-digestion of pAd5F35-CTL.Th with *I-CeuI/Pi-SceI* produced the two expected fragments. Digestion of pAd5F35-CTL.Th with *XhoI* also generated seven expected fragments (Fig. 3A and B). The correct insertion of the CTL.Th gene was further confirmed by sequencing.

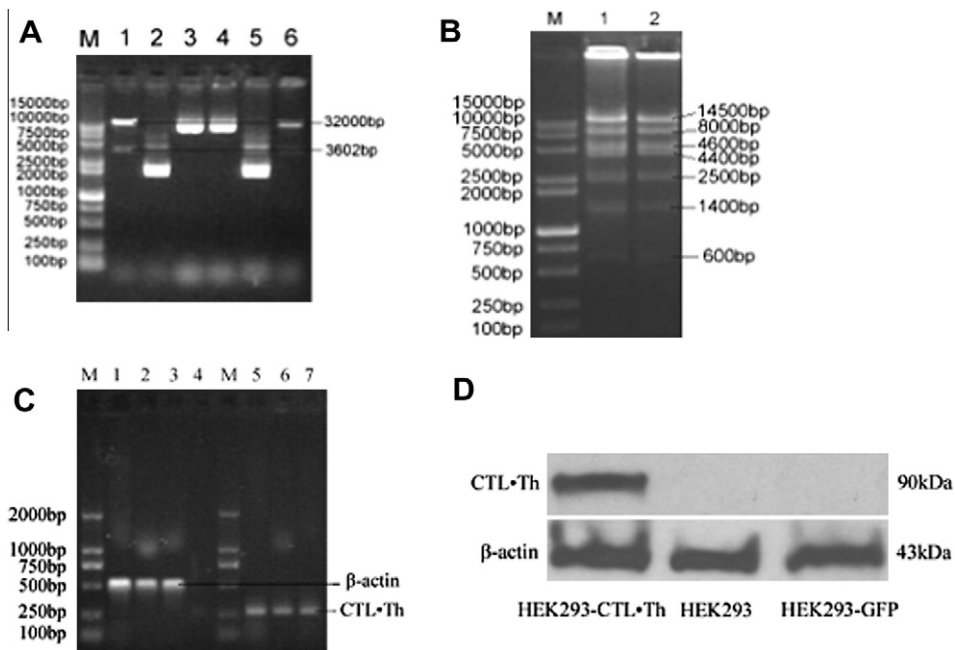
### 3.3. Generation and characterization of the Ad5F35-CTL.Th recombinant adenovirus vaccine

The recombinant pAd5F35-CTL.Th vector, and the control pAd5F35-GFP, were both packaged into HEK293 cells, and emerging recombinant adenoviruses Ad5F35-CTL.Th and Ad5F35-GFP were cultivated. In both cases a normal CPE was observed compared with uninfected HEK293 cells. Green fluorescence was observed in HEK293-GFP cells, indicating the efficient transfection and gene expression (Fig. 4A–F). Recombinant adenovirus was harvested from the transfected cells, with the Ad5F35-CTL.Th titer measured as  $2.5 \times 10^9$  PFU. Successful PCR amplification of recombinant virions DNA confirmed the inclusion of CTL.Th sequence into recombinant adenovirus virions.

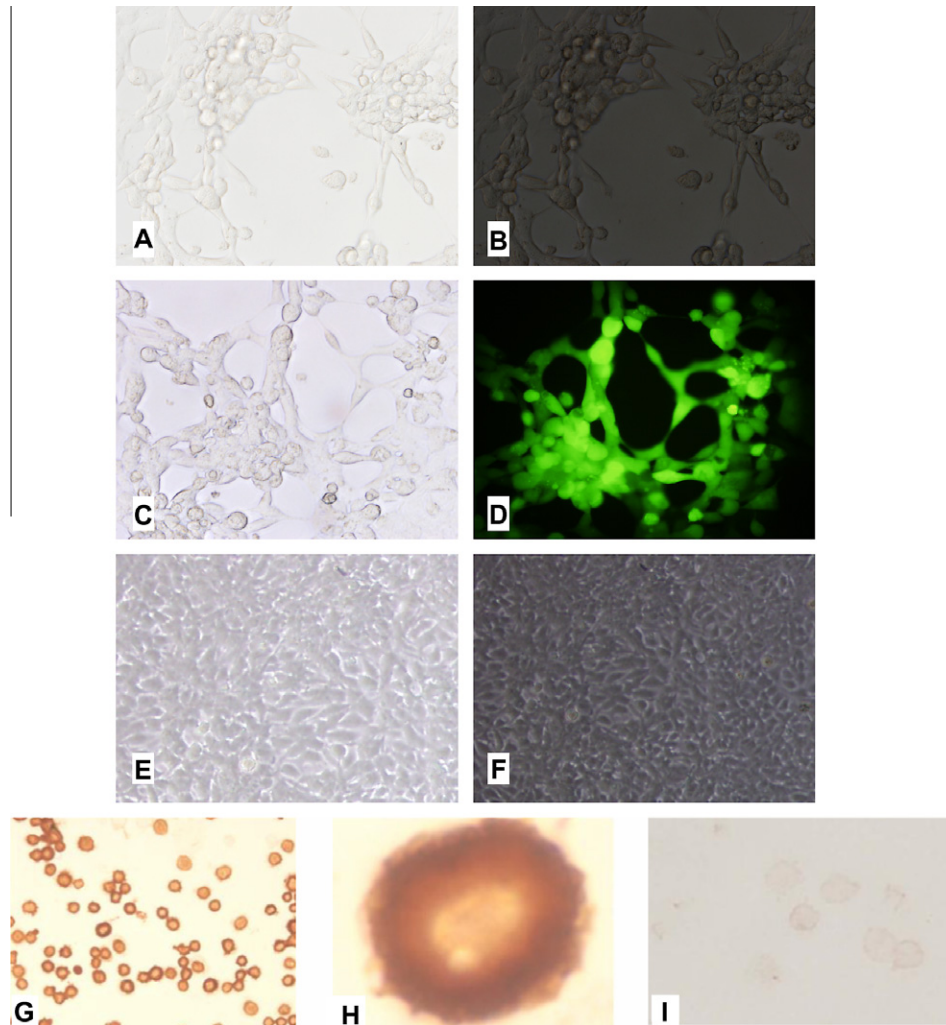
CTL.Th expression in HEK293-CTL.Th was further analyzed by RT-PCR and the amplified fragment was expected (Fig. 3C). CTL.Th protein expression was analyzed by Western blotting using antibody directed against the 6×His tag. The band at 90 kDa, corresponding to the predicted size of the recombinant CTL.Th protein, was observed in extracts of HEK293-CTL.Th cells, but not in parental HEK293 cells or HEK293-GFP cells (Fig. 3D).

### 3.4. Ad5F35-CTL.Th recombinant adenovirus efficiently transfected PBMCs and had little cytopathic activity

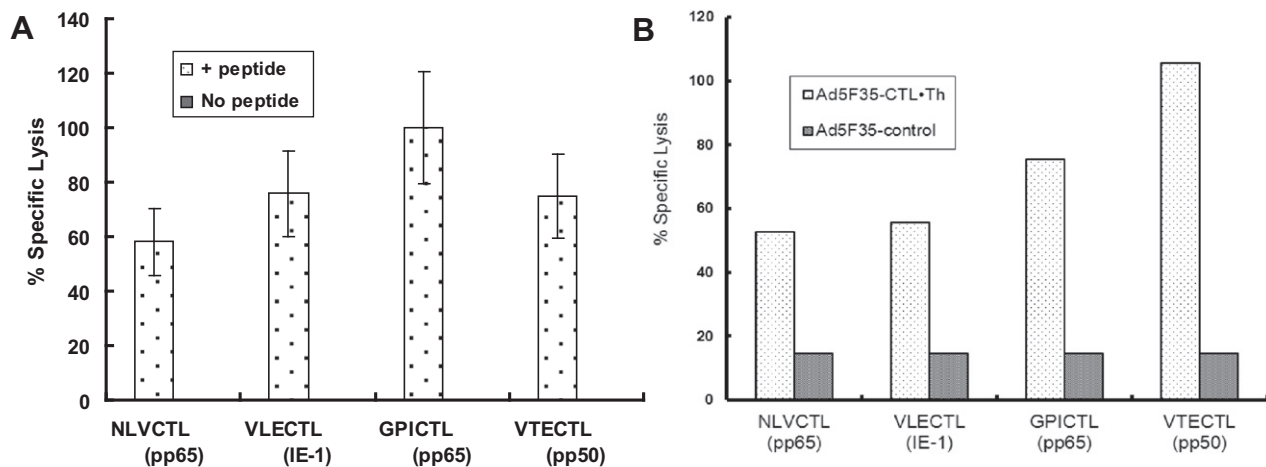
Strong expression of CTL.Th antigen was found in PBMCs cells infected with Ad5F35-CTL.Th, while no expression was detected in PBMCs infected with control Ad5F35-GFP. The typical pattern of staining indicated that the recombinant CTL.Th polyepitope or its proteasomal cleaved epitopes might both be localized in the cell membrane and cytoplasm (Fig. 4G–I). Expression of CTL.Th was time-dependent, and strongest expression was observed on day 6. No significant cytopathic activity was detected in infected cells, as cell viability was not significantly affected by Ad5F35-CTL.Th infection, with 90% of cells remaining viable.



**Fig. 3.** Generation and characterization of the Ad5F35-CTL.Th recombinant adenovirus vaccine. (A) Plasmid pAd5F35-CTL.Th digested with *I-CeuI/Pi-SceI*. Lanes 1 displays the expected restriction fragments generated from pAd5F35-CTL.Th (3.6 Kb + 32 Kb). (B) *XhoI* restriction enzyme digestion of recombinant pAd5F35-CTL.Th. Lanes 1 and 2 contain the 7 expected restriction fragments (14.5, 8.0, 4.6, 4.4, 2.5, 1.4 and 0.6 Kb). (C) RT-PCR result of CTL.Th polyepitope sequences from infected HEK293 cells. Lanes 5–7 show the CTL.Th PCR product at 245 bp; lanes 1–3 display the control  $\beta$ -actin PCR product at 578 bp. (D) CTL.Th polyepitope expression result by Western blotting using antibody directed against the 6×His tag. HEK293-CTL.Th cells present the anticipated CTL.Th polyepitope (90 kDa) band; no band was found in HEK293-GFP cells or parental HEK293 cells.



**Fig. 4.** The results of cytopathogenic effect in HEK293 cells and protein expression in PBMCs cells after infection. Following transfection of HEK293 cells a normal cytopathogenic effect was observed for HEK293-CTL.Th (A, bright field; B, dark field) and HEK293-GFP control cells (C, bright field; D, dark field), while no CPE was observed in uninfected HEK293 cells (E, bright field; F, dark field); in D green fluorescence is observed. G–I, Immunohistochemical detection of CTL.Th polyepitope expression in PBMCs infected with Ad5F35-CTL.Th (G and H) or in control cells infected with Ad5F35-GFP (I). Cells were stained with anti-6×His antibody to detect expression of the CTL.Th polyepitope which contains an amino-terminal 6×His tag. Cells in panel H were shown at a higher magnification to demonstrate the presence of antigen in both cell membrane and cytoplasm.



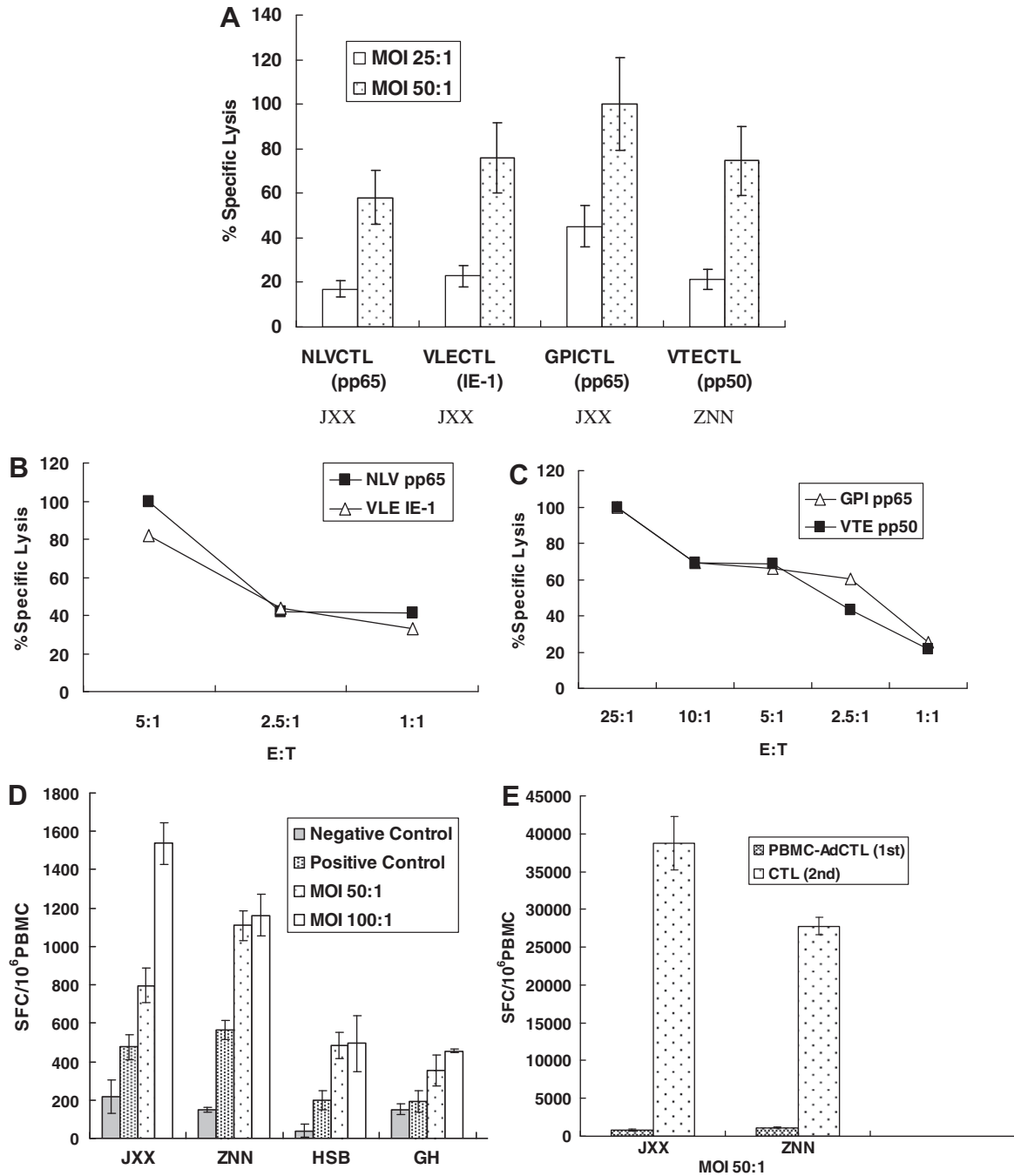
**Fig. 5.** Endogenous processing of HCMV CTL epitopes encoded by Ad5F35-CTL.Th. (A) HCMV epitope-specific lysis by HLA A2 (NLV and VLE), HLA A1-restricted (VTE), and A11-restricted (GPI CTL) CTL lines derived from two different healthy virus carriers. Peptide-sensitized and uncoated HLA-matched LCLs were used as target cells in the CTL assay. (B) HLA-matched LCL cell were infected (MOI 25:1) with either Ad5F35-CTL.Th or control adenovirus (referred to as Ad5F35-control) for 16–18 h and then exposed to HCMV-specific CTL lines. LCLs infected with adenovirus vectors were used as targets for NLV, VLE, VTE, and GPI -specific CTL lines. An E:T ratio of 2.5:1 was used for both assays.



3.5. HCMV CTL epitopes encoded by Ad5F35-CTL.Th is endogenously processed and presented

HCMV-specific CTL lines were generated from healthy virus carriers and their specificity was confirmed by their ability to lyse

HLA-matched target cells coated with the respective epitopes (Fig. 5A). HLA-matched LCLs infected with Ad5F35-CTL.Th (MOI 25:1) were also efficiently recognized by individual HCMV-specific CTL lines, whereas target cells infected with control Ad5F35 were not recognized (Fig. 5B).



**Fig. 6.** Activation and expansion of HCMV-specific CTL responses following stimulation with Ad5F35-CTL.Th. (A) PBMCs from the healthy virus carriers JXX (HLA A2,A11,DR4,DR15) and ZNN (HLA A1,B44,B35,DR7,DR11) were co-cultured with autologous PBMCs infected with Ad5F35-CTL.Th (MOI 50:1 or 25:1) at a responder to stimulator ratio of 2:1. On day 7, the cultures were restimulated with Ad5F35-CTL.Th -infected (MOI 50:1), x-irradiated autologous LCLs, and the growth medium was supplemented with rIL-2 (20 U/ml). Epitope-specific T cell reactivity was subsequently assessed on day 10 using in vitro cytotoxicity assays. Autologous LCLs coated with individual peptide epitopes (20 µg/ml) or uncoated were used as targets for these assays with an E:T ratio of 10:1. (B, C) Ad5F35-CTL.Th was highly efficient in stimulating multiple CTL responses, and strong cytolytic activity against four different epitopes (VTE, HLA A1-restricted; NLV and VLE, HLA A2-restricted; GPI, HLA A11-restricted) was observed at an E:T ratio as low as 1:1. (D) PBMCs from four healthy virus carriers JXX (A2, A11, DR4, DR15), ZNN (A1, B44, B35, DR7, DR11), HSB (A1, A2, B35, DR4, DR13) and GH (A1, A2, B8, DR4, DR9) were used in this assay. Unstimulated PBMCs were used as negative controls, while sensitized PBMCs with peptide epitope (GPISGHVLK for JXX, VTEHDTLLY for ZNN, NLVPMVATV for HSB, VLEETSVML for GH) were positive controls. PBMCs infected with Ad5F35-CTL.Th at MOI 100:1 or 50:1 served as the experimental group. The secretion of IFN-γ was tested. The results show that IFN-γ-producing cells has been largely induced from PBMCs stimulated by Ad5F35-CTL.Th. The results were expressed as mean ± SE of spot forming cells (SFC) per 10<sup>6</sup> PBMCs from four individual volunteers. (E) Effects of single or double Ad5F35-CTL.Th stimulation on the activation of epitope-specific CTLs. PBMCs from healthy donors (JXX and ZNN) stimulated either once or twice with Ad5F35-CTL.Th are shown. Epitope-specific T cell reactivity was assessed using ELISPOT assays.

### 3.6. Activated HCMV-specific CTLs stimulated with Ad5F35-CTL.Th show strong cytolytic activity

PBMCs from healthy virus carriers were stimulated with autologous PBMCs infected with Ad5F35-CTL.Th. It showed that a single stimulation was highly efficient to rapidly expand T cells, and these cells exhibited a strong HCMV epitope-specific cytolytic activity. CTLs expanded from two different donors showed reactivity to more than one epitope derived from different antigens. For example, T cell cultures from donor JXX showed strong cytolytic activity against 3 epitopes coming from two antigens (IE-1, pp65); while donor ZNN demonstrated cytolytic activity against 1 epitope coming from pp50 antigen (Fig. 6A). Moreover, the two different MOI (50:1 and 25:1) showed comparable levels of T cell stimulation.

The efficacy of Ad5F35-CTL.Th was also confirmed by testing the expanded T cell populations at different effector to target (E:T) ratios. Ad5F35-CTL.Th was highly efficient in stimulating multiple CTL responses, with strong cytolytic activity against four different epitopes (VTE, HLA A1-restricted; NLV and VLE, HLA A2-restricted; and GPI, HLA A11-restricted) observed at an E:T ratio as low as 1:1 (Fig. 6B and C). Moreover, the cytolytic activity was also elevated with the increase of E:T ratio.

### 3.7. CTL activity is accompanied by IFN- $\gamma$ release after Ad5F35-CTL.Th stimulation

PBMCs stimulated with Ad5F35-CTL.Th produced higher IFN- $\gamma$  amounts than controls. Ad5F35-CTL.Th efficiently sensitized PBMCs to antigen-specific CTLs. In addition, the two different MOI (100:1 and 50:1) showed comparable levels of IFN- $\gamma$  production, with higher IFN- $\gamma$  production observed at MOI 100:1 than MOI 50:1 especially for the donor JXX (Fig. 6D). Furthermore, IFN- $\gamma$  production cells in PBMCs stimulated with Ad5F35-CTL.Th (stimulated once) was lower than that in CTLs restimulated with Ad5F35-CTL.Th (stimulated twice), indicating stronger HCMV epitope-specific cytolytic activity upon twofold stimulation (Fig. 6E).

## 4. Discussion and conclusion

HCMV disease remains a major obstacle for allogeneic BMT and prevention of HCMV infection by a vaccine has become a high-priority goal (Schleiss, 2008). Adoptive transfer of HCMV-specific CTL cells into transplant recipients has demonstrated therapeutic benefits in relation to HCMV diseases (Hebart and Einsele, 2004; Peggs et al., 2003). A vaccine that can induce a broad repertoire of HCMV-specific immune responses in different ethnic populations with various HLA alleles is likely to provide more effective protection against HCMV-associated pathogenesis.

Since the Chinese population constitutes approximately one fifth of the world population and has extremely polymorphic HLA alleles, it is essential to develop HCMV epitope vaccines more specific to the Chinese to prevent worldwide HCMV infection. In this context and based on our forecasting system, we identified the combination of 14 HLA allele sites (A2, A24, A1, A3, A11, A68, B44, B7, A23, A26, B35, B38, B8 and B27), and the CPF of 14 HLA allele sites in the Chinese population reached 92.1%. Then, according to these selected HLAs and literature data on HLA-restricted HCMV epitopes, we designed the novel chimeric vaccine which encodes 76 HCMV CTL epitopes and 7 Th epitopes as a polyepitope.

Optimized selection of epitopes can precisely direct the evoked immune response at conserved and highly immunogenic regions of several antigens. Therefore, in addition to the coverage of HLA alleles in a given population, a good epitope-based vaccine also takes into account coverage of epitopes, coverage of HLA/antigen combinations, and the probability of each epitope to be properly cleaved

in the sequence. HCMV has 165 open reading frames (ORFs), and accumulating evidence has shown that HCMV specific immune responses are not only restricted to gB, pp65 and IE-1 antigens, but also are directed towards more than 70% of the HCMV reading frames (Elkington et al., 2004; Manley et al., 2004; Schleiss, 2008; Sylwester et al., 2005) which includes “non-essential” genes encoding modulators of adaptive immune responses, such as US2, US3, US6, US11. In this study, computational methods such as CPF algorithm, SYFPEITHI algorithm and MAPPP algorithm for predicted as well as experimentally determined epitopes were incorporated into the selection of the optimal polyepitope. These epitopes included in this polyepitope were derived from 15 different viral proteins involved in different stages of viral infection, all of which significantly reduced any potential threat of immune escape from the selective expression of antigens.

As described above, vaccines incorporating various antigens expressed in all reproduction phase of virus would provide a strategy to immunize against HCMV infection clinically. To circumvent allele specificity for the required Th epitope, a series of Th sequences that promiscuously bind to HLA II alleles have been evaluated in combination with the polyepitope. In our study, the HCMV epitopes encoded by this polyepitope were endogenously expressed and processed by human cells. We found that stimulation with Ad5F35-CTL.Th rapidly expanded multiple antigen-specific human CTL cells from healthy virus carriers *in vitro*, and these CTL cells exhibited a strong HCMV epitope-specific cytolytic activity against HLA restricted target cells. IFN- $\gamma$ -producing cells were largely induced from PBMCs stimulated by Ad5F35-CTL.Th or expanded antigen-specific CTL, which indicated T cell reactivation. The data presented here emphasize the strength and adaptability of the polyepitope-based approach for clinical translation and may lead to significant advances in the application of adoptive immunotherapy to a wide range of diseases. It will be useful for HCMV vaccine development for the BMT patients.

In the development of a new HCMV vaccine, it is important that the vaccine has high immunogenicity, low production cost, and low pathogenicity. Compared with traditional adenoviral vectors, Ad5F35 used in this study is being increasingly recognized for its high efficiency and low toxicity and has been used in multiple human gene therapy clinical trials. The Ad5F35 vector contains a chimeric fiber gene encoding the fiber tail of Ad5 in combination with the fiber shaft and knob domains of Ad35. Cell entry of Ad5F35 is thought to be mediated by CD46, a receptor expressed on most human cells (Gaggar et al., 2003). Liver tropism and hepatotoxicity are significantly lower for Ad5F35 than for Ad5 (Xin et al., 2005). Importantly, Ad5F35 infection does not induce acute responses or the production of proinflammatory cytokines. Ad5F35 has been used as a vaccine delivery vehicle in other infectious disease models including hepatitis C (Thammanichanon et al., 2008) and HIV (Xin et al., 2007), where potent transgene product-specific antibody and T-cell responses were reported. In terms of efficacy, Ad5F35 appears to be more effective than other comparable vectors for recombinant protein expression in hematopoietic cells (Yotnda et al., 2001). In this study we successfully constructed a novel HCMV vaccine based on the replication-deficient Ad5F35 that encodes HLA-specific HCMV polyepitope nucleotide vaccine. *Ex vivo* infecting PBMCs with Ad5F35-CTL.Th consistently demonstrated high expression of CTL.Th polyepitope antigen and low toxicity, which in turn resulted in strong stimulation of antigen specific CTLs. Thus, combination of the Ad5F35 expression system with polyepitope technology provides a potential strategy for the expansion of T cells.

In summary, we have developed a recombinant adenovirus vaccine against HCMV based on Ad5F35 expression system and polyepitope technology, which covers 92% of the Chinese population. Stimulation with this adenovirus vector expressing HCMV

polyepitope is capable of inducing multiple, independent HLA-restricted CTL responses. Therefore, this vaccine may be a promising candidate for clinical trials and in prime-boost strategies in combination with other HCMV vaccine formulations, and we might also use this approach to develop suitable vaccines for the non-Chinese population.

### Conflict of interest

There is no conflict of interests for each author.

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