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Identification of conserved and HLA-A*2402-restricted epitopes in Dengue virus serotype 2

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ABSTRACT

In this study, we set out to identify dengue virus serotype 2 (DENV-2)-specific HLA-A*2402-restricted epitopes and determine the characteristics of T cells generated to these epitopes. We screened the full-length amino-acid sequence of DENV-2 to find potential epitopes using the SYFPEITHI algorithm. Twelve putative HLA-A*2402-binding peptides conserved in hundreds of DENV-2 strains were synthesized, and the HLA restriction of peptides was tested in HLA-A*2402 transgenic mice. Nine peptides ($NS4b_{228–237}$, $NS2a_{73–81}$, $E_{298–306}$, $M_{141–149}$, $NS4a_{96–105}$, $NS4b_{159–168}$, $NS5_{475–484}$, $NS1_{162–171}$, and $NS5_{611–620}$) induced high levels of peptide-specific IFN-γ-secreting cells in HLA-A*2402 transgenic mice. Apart from IFN-γ, $NS4b_{228–237}$-, $NS2a_{73–81}$- and $E_{298–306}$-specific $CD8^+$ cells produced TNF-α and IL-6 simultaneously, whereas $M_{141–149}$- and $NS5_{475–484}$- $CD8^+$ cells produced only IL-6. Moreover, splenic mononuclear cells (SMCs) efficiently recognized and killed peptide-pulsed splenocytes. Furthermore, each of nine peptides could be recognized by splenocytes from DENV-2-infected HLA-A*2402 transgenic mice. The SMCs from HLA-A*2402 transgenic mice immunized with nine immunogenic peptides efficiently killed DENV-2-infected splenic monocytes. The present identified epitopes have the potential to be new diagnostic tools for characterization of T-cell immunity in DENV infection and may serve as part of a universal epitope-based vaccine.

1. Introduction

Dengue virus (DENV) is a single-stranded positive RNA virus that includes four different serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) that differ in primary amino acid sequence by 30–35%. The genome encodes three structural proteins (C, M, and E) and seven nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5). Every year, there are 50–100 million cases of dengue fever (DF), including more than 500,000 cases of dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) (Steidel et al., 2012). It is estimated that 2.1 million cases of DHF/DSS occur every year, resulting in 21,000 deaths (Callaway, 2007). DENV is transmitted by an infected mosquito, and infections have become a major threat to public health in numerous tropical and subtropical countries and regions (Duangchinda et al., 2010). Various strategies have been used to develop a vaccine for dengue fever, such as live attenuated vaccines, inactivated vaccines, recombinant subunit vaccines, virus vectored and viral-like particle-based vaccines, and DNA vaccines (McArthur et al., 2013). However, to date, there is no efficacious antiviral therapy or licensed vaccine available.

At present, the role of DENV-specific $CD8^+$ T cells in DENV infection and subsequent disease manifestations is not well understood. Early studies suggested that DENV-specific $CD8^+$ T cells may have pathogenic roles in DENV infection (An et al., 2004; Mathew and Rothman, 2008). However, in the past several years, increasing evidences has indicated that DENV-specific $CD8^+$ T cells are likely to play significant roles in controlling and clearing

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DENV infection. Initially, some research studies have demonstrated that DENV-specific $CD8^+$ T cells display lytic activity against DENV-infected cells (Imrie et al., 2007; Yauch et al., 2009). Subsequently, researchers found that DENV-specific $CD8^+$ T cells play a crucial role in controlling DENV replication and infection by secreting interferon (IFN)-γ (Yauch et al., 2010; Weiskopf et al., 2011). A recent study also showed that epitope-specific $CD8^+$ cytotoxic T lymphocytes (CTLs) can recognize and lyse peptide-pulsed cells, as well as DENV-infected cells (Testa et al., 2012). Furthermore, a more recent study suggested that a vigorous response by multifunctional $CD8^+$ T cells is associated with protection from dengue virus disease (Weiskopf et al., 2013).

In general, further studies of DENV-specific $CD8^+$ T cells are necessary to improve understanding of the contributions of $CD8^+$ T cells to dengue control. A detailed understanding of a protective $CD8^+$ T-cell response following DENV infection will greatly enhance the development and evaluation of an efficacious DENV vaccine. To best determine the characteristics of $CD8^+$ T cells in protection, future work is needed to identify and characterize a large number of $CD8^+$ T-cell epitopes. Such knowledge would greatly enrich understanding of the role of $CD8^+$ T cells in DENV infection and facilitate future development of effective vaccines targeting the $CD8^+$ T-cell epitopes.

Given the high prevalence of the HLA-A*2402 allele in most populations, irrespective of gender and race, we proceeded to identify HLA-A*2402-restricted epitopes in DENV-2. In the present study, the amino acid sequences of DENV-2 were analyzed by predictive algorithm for screening of putative HLA-A*2402-binding peptides. The HLA restriction of the predicted peptides was tested in HLA-A*2402 transgenic mice, and the characteristics of peptide-specific T cells were determined by IFN-γ ELISPOT assay, intracellular cytokine staining (ICS) assay, and in vitro cytotoxicity assay.

2. Materials and methods

2.1. Epitope prediction and peptide synthesis

The T-cell epitope prediction algorithm (SYFPEITHI, http://www.syfpeithi.de/Scripts/MHCServer.dll/EpitopePrediction.htm) and the proteasomal cleavage prediction server (PAProc, http://www.paproc.de/) were used to screen the complete amino acid sequences of DENV-2 (New Guinea C strain; GenBank Accession No.: AAC59275.1) for potential HLA-A*2402-restricted $CD8^+$ T-cell epitopes. Peptides (epitope candidates) were synthesized by a peptide company (ChinaPeptides Co., Ltd. Shanghai, China) and their purity (>95%) was ascertained by mass spectrometry.

2.2. Dengue virus, cell line, and mice

DENV-2 (New Guinea C strain) and C6/36 cells were purchased from ATCC (Manassas, VA, USA). CB6F1-Tg (HLA-A*2402/H2-Kb)A24.01 mice (HLA-A*2402 transgenic mice) were purchased from Taconic Corporation (Taconic Farms Inc, New York, USA) and bred under specific-pathogen-free conditions. Female C57BL/6 mice (6–8 weeks) were provided by the Laboratory Animal Center of Wenzhou Medical University.

2.3. Immunization of transgenic mice with peptide

Female HLA-A*2402 transgenic mice (4–6 weeks of age) were subdivided into fourteen groups (5 mice/group). 250 μg peptide in 250 μl PBS were emulsified in 250 μl Complete Freund's Adjuvant (CFA), and the mixture was inoculated subcutaneously (s.c.) into the back skin of transgenic mice (50 μg peptide/mouse). One week later, the mixture, the same peptide emulsified in Incomplete Freund's Adjuvant (IFA), was used to boost mice three additional times at weekly intervals. In addition, the mix of multiple immunogenic peptides (50 μg/peptide) was used to immunize transgenic mice according to the above-mentioned immunization protocol. Control mice (mock-immunized mice) received CFA and IFA without peptide. One week after the last immunization, splenocytes and lymph node cells (deltopectoral and popliteal lymph nodes) from mice immunized with single peptide were used for murine IFN-γ ELISPOT assay. Splenic mononuclear cells (SMCs) were isolated from splenocytes by gradient centrifugation using leukocyte separation medium. The SMCs were used for intracellular cytokine staining (ICS) assay and LDH-based cytotoxicity assay. Splenocytes and SMCs from mice immunized with multiple immunogenic peptides were used for murine IFN-γ ELISPOT assay and LDH-based cytotoxicity assay, respectively. Protocols for the animal experiments were approved by the Animal Ethics Committee of Wenzhou Medical University.

2.4. Infection of transgenic mice with DENV-2

C6/36 cells were inoculated with DENV-2 for 2 h at 37 °C and then cultured at 28 °C. The virus-containing media was harvested 3–5 days after infection and filtered through a 0.22-μm filter.

HLA-A*2402 transgenic mice (5 weeks old) were intravenously injected with 1×10^7 PFU (per mouse) of DENV-2 via the tail vein. Four weeks after infection, the splenocytes were isolated from the spleen and used for the murine IFN-γ ELISPOT assay. Protocols for the animal experiments were approved by the Animal Ethics Committee of Wenzhou Medical University.

2.5. Preparation of target cells

Splenocytes were isolated from the spleen of HLA-A*2402 transgenic mice and treated with ice-cold Na_2HPO_4/citric acid buffer (a mixture of equal volumes of 0.123 M Na_2HPO_4 and 0.263 M citric acid, pH3.2) for 90 s as described previously (Petersen et al., 2009). Citric acid-treated cells were incubated for 3 hours at 4 °C in the presence or absence of individual peptide. Both splenocytes loaded with no peptide and splenocytes loaded with peptide served as target cells in LDH-based cytotoxicity assay. In addition, splenocytes of HLA-A*2402 transgenic mice were seeded into the wells of six-well plates. After a 2-h incubation at 37 °C, the supernatants were removed and the adherent cells (splenic monocytes) were collected. Subsequently, splenic monocytes were infected with DENV-2 at a multiplicity of infection (MOI) of 5. After a 2-h infection at 37 °C, the cells were resuspended in RPMI-1640 medium and cultured at 37 °C, 5% CO_2 incubator. After 24 h of incubation, both non-infected splenic monocytes and DENV-2-infected splenic monocytes served as target cells in LDH-based cytotoxicity assay.

2.6. Murine IFN-γ ELISPOT assay

Murine IFN-γ ELISPOT assays were performed in pre-coated 96-well plates (U-CyTech Company, Utrecht, the Netherlands). Murine splenocytes and lymph node cells from peptide-immunized mice and DENV-2-infected mice were dispensed at a predetermined density of 2×10^6 cells/ml in duplicate wells (2×10^5 cells/well). Test wells had cells and peptide (20 μg/ml), whereas background wells contained cells but no peptide. The plate was incubated at 37 °C/5% CO_2 for 24 h. Subsequently, the plate was processed according to the manufacturer's instructions. The colored spots, representing epitope-specific IFN-γ-producing T cells (spot-forming cells, SFCs), were counted by using an automatic ELISPOT reader (Beijing Sage

Table 1
Dengue virus serotype 2-derived putative HLA-A*2402-binding peptides.

Peptides	Sequences	HLA restriction[a]	SYFPEITHI Predictive scores	Serotypes[b]
$NS4b_{228–237}$	SYLAGAGLLF	HLA-A*2402	24	DENV-2 (770)
$NS2a_{73–81}$	TYLALLAAF	HLA-A*2402	23	DENV-2 (770)
$NS3_{471–479}$	QYIYMGEPL	HLA-A*2402	23	DENV-2 (800)
$E_{298–306}$	SYSMCTGKF	HLA-A*2402	22	DENV-2 (1500)
$M_{141–149}$	AYTIGTTHF	HLA-A*2402	21	DENV-2 (770)
$NS1_{33–42}$	KFQPESPSKL	HLA-A*2402	21	DENV-2 (780)
$NS4a_{96–105}$	WYAQIQPHWI	HLA-A*2402	21	DENV-2 (760)
$NS4b_{159–168}$	PYDPKFEKQL	HLA-A*2402	21	DENV-2 (790)
$NS5_{475–484}$	WYMWLGARFL	HLA-A*2402	20	DENV-2 (720)
$NS1_{162–171}$	VFTTNIWLKL	HLA-A*2402	20	DENV-2 (650)
$NS5_{611–620}$	TFTNMEAQLI	HLA-A*2402	19	DENV-2 (760)
$NS5_{562–571}$	KKLAEAIFKL	HLA-A*2402	17	DENV-2 (760)

[a] The peptide is potentially restricted by the following HLA allele.
[b] Peptide is conserved in DENV-2 (the digits in brackets are the numbers of DENV-2 isolates).

Creation Science Co. Ltd, Beijing, China). The frequency of peptide-specific T cells was expressed as IFN-γ SFCs/2×10^5 cells.

2.7. Intracellular cytokine staining assay

Secretion of TNF-α and IL-6 by $CD8^+$ cells from peptide-immunized mice was detected using ICS assay. In brief, SMCs (1×10^6 cells in 0.5 ml medium) from mice immunized with single peptide were seeded into the wells of a six-well plate and cultured in the presence or absence of peptide (20 μg/ml). After 1 h, Brefeldin A (BFA, 10 mM, Enzo Biochem Inc, NY, USA) was added to the cells. After 6 h of incubation at 37 °C, cells were washed with PBS and then fixed with 4% paraformaldehyde for 10 min at 4 °C. Cell were permeabilized with 0.5% saponin for 15 min at 4 °C and stained with FITC-conjugated anti-mouse CD8a monoclonal antibodies (mAb), APC-conjugated anti-mouse TNF-α mAb and PE-conjugated anti-mouse IL-6 mAb (eBioscience Company, USA) for 1 h at 4 °C. Cells were analyzed by flow cytometry (BD Bioscience, USA). The percentages of $CD8^+$ TNF-α^+ cells or $CD8^+$ IL-6^+ cells in total $CD8^+$ cells were measured.

2.8. LDH-based cytotoxicity assay

The Lactate dehydrogenase (LDH) Cytotoxicity Assay kit (Promega, Wisconsin, USA) was used to detect the cytotoxic activity of peptide-specific cytotoxic T lymphocyte (CTL) against peptide-pulsed splenocytes and DENV-2-infected splenic monocytes. The test was performed in a V-bottom 96-well culture plate according to the manufacturer's instructions. The SMCs from mice immunized with single peptide or mice immunized with multiple immunogenic peptides worked as effector cells, and citric acid-treated splenocytes loaded with no peptide or peptide, non-infected splenic monocytes, and DENV-2-infected splenic monocytes served as target cells. Experimental wells contained varying numbers of effector cells and a constant number of target cells (1×10^4 target cells/well), and the effector to target cell ratios (E:T) were 10:1, 5:1, and 1:1. Except for these wells, the following wells were also set: Effector Cell Spontaneous LDH release (ES), Target Cell Spontaneous LDH Release (TS), Target Cell Maximum LDH Release (TM), Volume Correction (VC), and Culture Medium Background (CM). After a 9-h incubation at 37 °C, the plates were centrifuged at 400 g for 4 min, and an aliquot of 50 μl was taken to record the absorbance at 490 nm. The percent lysis of target cells for each E:T ratio was calculated using the values according to the following formula:

$$\%\,\text{cytotoxicity} = \frac{\text{experimental} - \text{ES} - \text{TS} + \text{CM}}{\text{TM} - \text{TS} - \text{VC} + \text{CM}} \times 100$$

2.9. Statistical analysis

Results were expressed as mean ± SEM. Student's *t*-test was used to determine the significant differences between the mean values of the experimental groups. $p < 0.05$ was considered significant.

3. Results

3.1. Epitope candidates

Epitope candidates were selected and synthesized according to the following criteria. First, the peptide had a high predictive score and a human proteasomal cleavage site (C terminus). Second, the sequence of the peptide was highly conserved in most DENV-2 strains. Twelve putative HLA-A*2402-binding peptides [$NS4b_{228–237}$(SYLAGAGLLF), $NS2a_{73–81}$(TYLALLAAF), $NS3_{471–479}$(QYIYMGEPL), $E_{298–306}$(SYSMCTGKF), $M_{141–149}$-(AYTIGTTHF), $NS1_{33–42}$(KFQPESPSKL), $NS4a_{96–105}$(WYAQIQPHWI), $NS4b_{159–168}$(PYDPKFEKQL), $NS5_{475–484}$(WYMWLGARFL), $NS1_{162–171}$(VFTTNIWLKL), $NS5_{611–620}$(TFTNMEAQLI), and $NS5_{562–571}$(KKLAEAIFKL)] were chosen and synthesized. Among twelve peptides, four peptides were nonapeptides, while eight peptides were decapeptides. BLAST results indicated that all peptides were conserved in more than 600 DENV-2 isolates, with $E_{298–306}$ being the mostly conserved peptide (Table 1).

3.2. Induction of peptide-specific CTL response in peptide-immunized HLA-A*2402 transgenic mice

HLA-A*2402 transgenic mice were immunized four times with single peptide and both splenocytes and lymph node cells were collected one week after the last immunization. ELISPOT and ELISA were used to determine the breadth of the T-cell response following peptide immunization. Among 12 potential HLA-A*2402-restricted peptides, 9 peptides ($NS4b_{228–237}$, $NS2a_{73–81}$, $E_{298–306}$, $M_{141–149}$, $NS4a_{96–105}$, $NS4b_{159–168}$, $NS5_{475–484}$, $NS1_{162–171}$, and $NS5_{611–620}$) induced substantial peptide-specific IFN-γ spot-forming cells (SFCs) in both splenocytes and lymph node cells detected by ELISPOT assay. However, $NS3_{471–479}$, $NS1_{33–42}$, and $NS5_{562–571}$ immunization did not induce significant IFN-γ-secreting T-cell immune response (Fig. 1). Among 9 immunogenic peptides, the highest frequency of SFCs was specific for peptide $NS5_{475–484}$ (181 ± 24 SFCs/2×10^5 splenocytes; 94 ± 7 SFCs/2×10^5 lymph node cells), while the lowest frequency of SFCs was directed to peptide $NS2a_{73–81}$(24 ± 7 SFCs/2×10^5 splenocytes; 16 ± 3 SFCs/2×10^5 lymph node cells). Next, in order to determine whether peptide-specific $CD8^+$ cells could produce

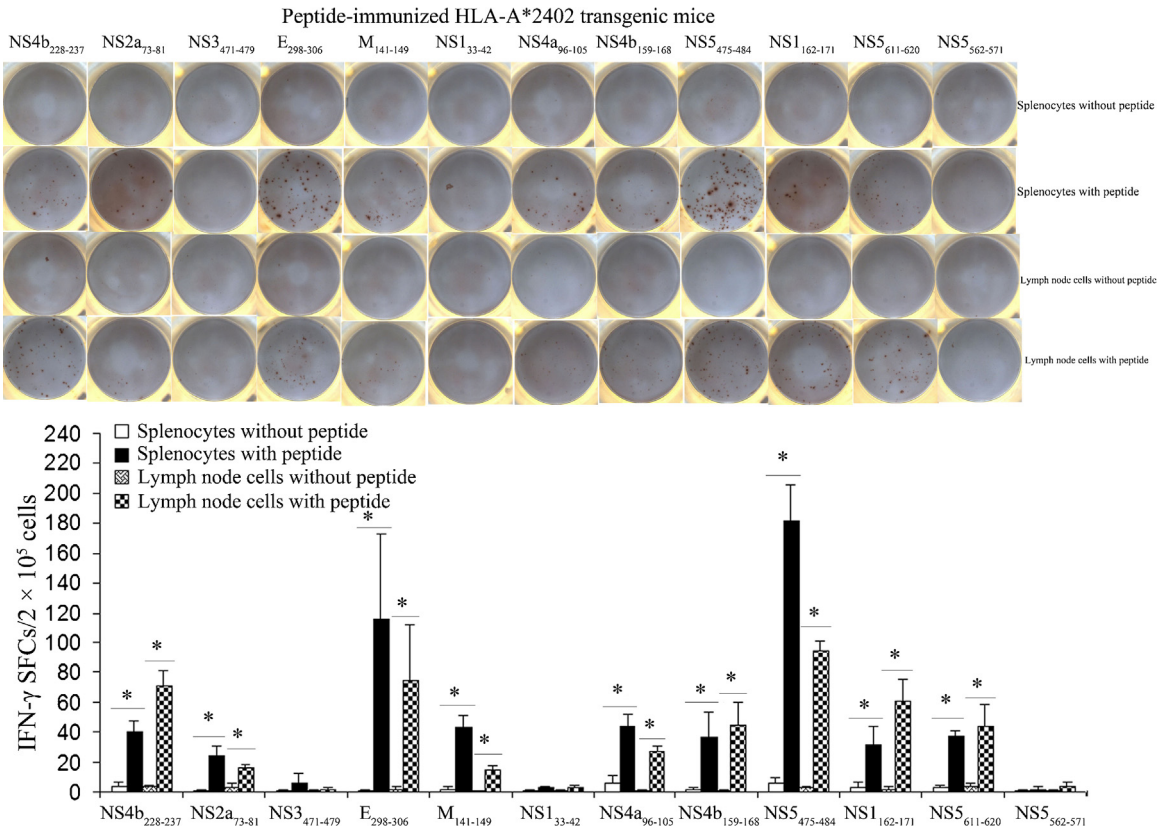

Fig. 1. Peptide-specific IFN-γ-producing cells in spleen and lymph nodes from peptide-immunized HLA-A*2402 transgenic mice. Splenocytes and lymph node cells were isolated from peptide-immunized mice and were restimulated in vitro with no peptide or the same peptide. The frequencies of IFN-γ SFCs/2×10^5 splenocytes or lymph node cells were detected using ELISPOT assay and presented as mean ± SD ($n = 5$). *The positive response to a peptide.

proinflammatory cytokines (TNF-α and IL-6), SMCs were stimulated with peptides, and ICS assay was used to detect the frequencies of $CD8^+$ TNF-α^+ cells or $CD8^+$ IL-6^+ cells in $CD8^+$ cells. $NS4b_{228–237}$, $NS2a_{73–81}$, and $E_{298–306}$ induced multifunctional $CD8^+$ cells simultaneously producing TNF-α and IL-6, whereas $M_{141–149}$- and $NS5_{475–484}$-specific $CD8^+$ cells produced only IL-6 (Fig. 2). It was also determined whether peptide-specific T cells have the ability to recognize and kill peptide-pulsed splenocytes. SMCs from peptide-immunized transgenic mice worked as effector cells, while splenocytes loaded with no peptide or peptide served as target cells. SMCs from immunogenic peptide-immunized mice efficiently killed peptide-pulsed splenocytes whereas that from other peptides-immunized mice displayed poor cytotoxic activity. Moreover, the percentage of peptide-pulsed splenocytes killed by effector cells is proportional to the effector to target cell ratio. The SMCs from $NS4a_{96–105}$-immunized mice showed the highest percent lysis of peptide-pulsed splenocytes (23.26 ± 14.13%, E:T ratio = 10:1; 16.55% + 6.31, E:T ratio = 5:1; 5.37% + 0.82, E:T ratio = 1:1) (Fig. 3).

3.3. Peptide-specific IFN-γ-secreting T cells in DENV-2-infected HLA-A*2402 transgenic mice

In order to determine whether DENV proteins could be degraded into these peptides in mouse cells, DENV-2 was used to infect HLA-A*2402 transgenic mice, and peptide-specific IFN-γ-secreting T cells were detected by ELISPOT assay. Nine immunogenic peptides ($NS4b_{228–237}$, $NS2a_{73–81}$, $E_{298–306}$, $M_{141–149}$, $NS4a_{96–105}$, $NS4b_{159–168}$, $NS5_{475–484}$, $NS1_{162–171}$, and $NS5_{611–620}$) were efficiently recognized by splenocytes from DENV-2-infected HLA-A*2402 transgenic mice, and the frequencies of IFN-γ-secreting T cells in 2×10^5 splenocytes were 38 ± 15 SFCs, 26 ± 11 SFCs, 30 ± 13 SFCs, 29 ± 14 SFCs, 21 ± 10 SFCs, 23 ± 6 SFCs, 9 ± 2 SFCs, 12 ± 4 SFCs, and 34 ± 11 SFCs, respectively (Fig. 4). For the remaining 3 peptides, there were no significant IFN-γ-secreting T cells in DENV-2-infected HLA-A*2402 transgenic mice.

3.4. Peptide-specific CTL in transgenic mice immunized with multiple immunogenic peptides

The mixture of nine immunogenic peptides ($NS4b_{228–237}$, $NS2a_{73–81}$, $E_{298–306}$, $M_{141–149}$, $NS4a_{96–105}$, $NS4b_{159–168}$, $NS5_{475–484}$, $NS1_{162–171}$, and $NS5_{611–620}$) was used to immunize HLA-A*2402 transgenic mice, and peptide-specific IFN-γ-secreting T cells were tested by ELISPOT assay. Each of the immunogenic peptides was able to elicit peptide-specific IFN-γ cells in splenocytes, similar to the results in mice immunized with single peptide. The frequencies of IFN-γ-secreting T cells in 2×10^5 splenocytes were 44 ± 7 SFCs, 28 ± 5 SFCs, 79 ± 12 SFCs, 54 ± 21 SFCs, 44 ± 13 SFCs, 24 ± 14 SFCs, 41 ± 12 SFCs, 28 ± 16 SFCs, and 22 ± 11 SFCs, respectively (Fig. 5). Moreover, the SMCs were able to efficiently kill DENV-2-infected splenic monocytes (84.87 ± 14.91%, E:T ratio = 10:1; 62.4 ± 8.55%, E:T ratio = 5:1; 36.2 ± 6.87%, E:T ratio = 1:1) (Fig. 6).

4. Discussion

Given that DENV-specific $CD8^+$ T cells may play an important role in limiting DENV infection, this study aimed to find novel HLA-A*2402-restricted epitopes in DENV-2. Previous studies have demonstrated that interferon gamma (IFN-γ) plays an important role in the clearance of DENV following infection (Diamond et al., 2000; Diamond and Harris, 2001) and mediates the

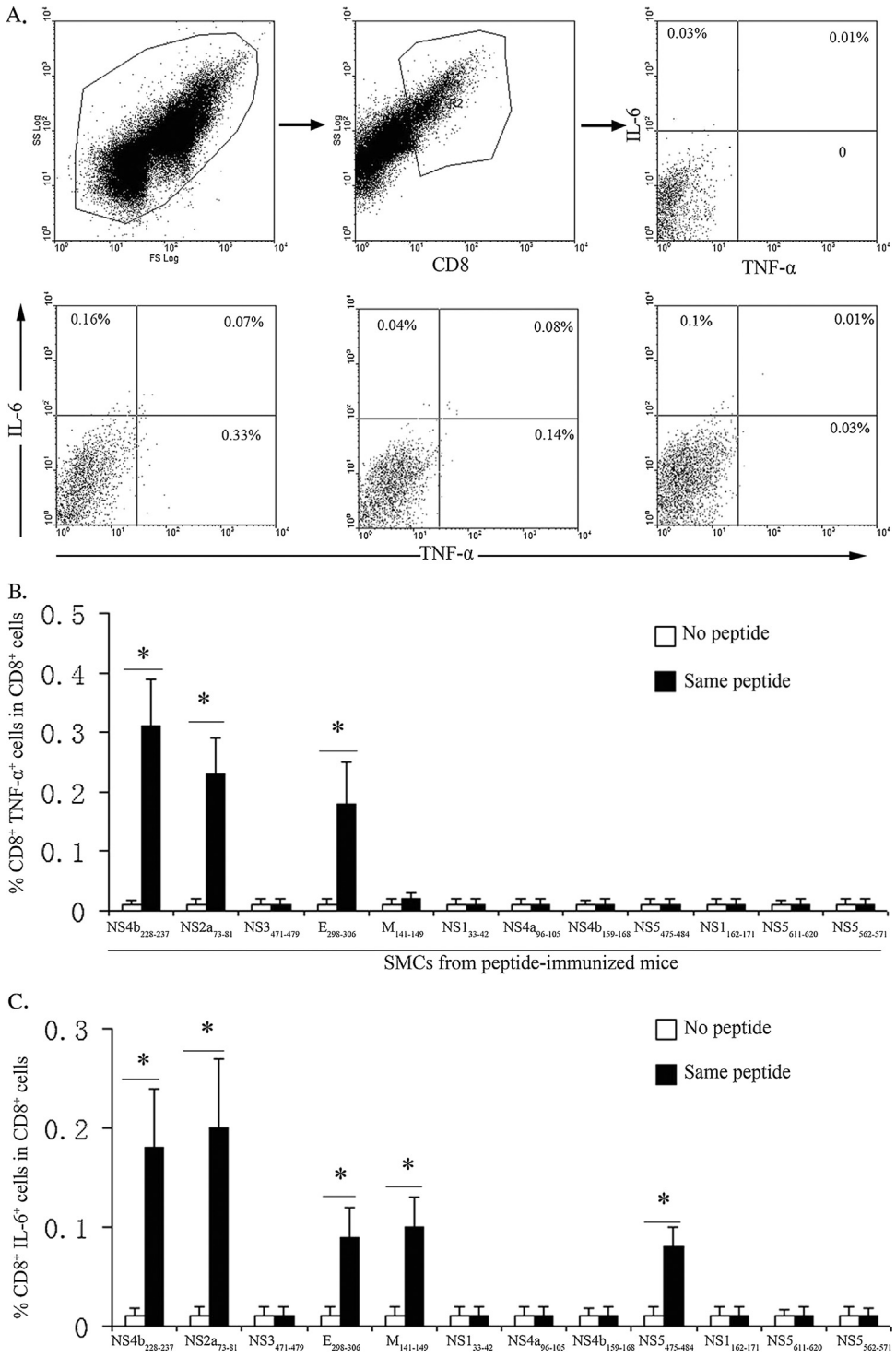

Fig. 2. Peptide-specific TNF-α- and Il-6-secreting cells in $CD8^+$ cells from peptide-immunized HLA-A*2402 transgenic mice splenic mononuclear cells (SMCs) were isolated from peptide-immunized mice and were restimulated in vitro with no peptide or the same peptide as described in methods. The percentages of $CD8^+$ $TNF\text{-}\alpha^+$ cells (B) or $CD8^+$ $IL\text{-}6^+$ cells (C) in total $CD8^+$ cells were determined by ICS assay and presented as mean ($n = 5$) (A). The representative figures of ICS assay. *The positive response to a peptide.

protective role of $CD8^+$ T cells (Yauch et al., 2009). Therefore, it was initially important to determine whether these epitope candidates could be presented by HLA-A*2402 molecules to T cells and induce the IFN-γ-secreting T-cell response in HLA-A*2402 transgenic mice. ELISPOT results showed that nine immunogenic peptides ($NS4b_{228–237}$, $NS2a_{73–81}$, $E_{298–306}$, $M_{141–149}$, $NS4a_{96–105}$, $NS4b_{159–168}$, $NS5_{475–484}$, $NS1_{162–171}$, and $NS5_{611–620}$) could elicit the IFN-γ-secreting T-cell response in both the spleen and lymph nodes of peptide-immunized transgenic mice. As expected, all twelve epitope candidates did not induce the significant peptide-specific IFN-γ-secreting T-cell response in both C57BL/6 mice and Balb/c mice (data not shown). In addition, there was no significant peptide-specific IFN-γ-secreting T-cell response in mock-immunized transgenic mice. All of these data confirm that these nine peptides are restricted by the HLA-A*2402 molecule and should be $CD8^+$ T-cell epitopes. In fact, apart from IFN-γ, both TNF-α

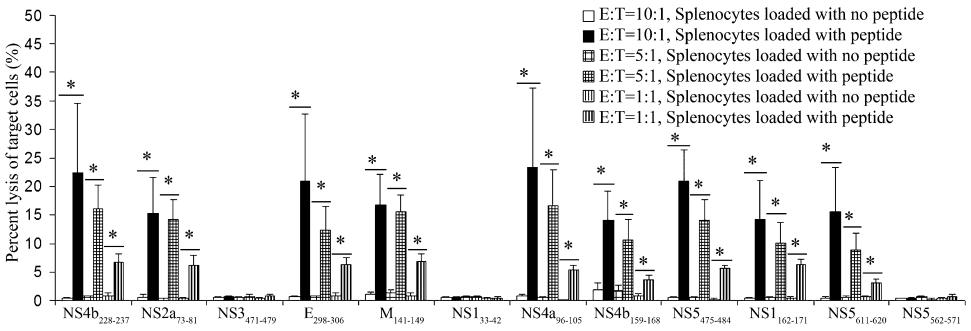

Fig. 3. The cytotoxic activity of splenic mononuclear cells from peptide-immunized HLA-A*2402 transgenic mice. Splenic mononuclear cells (SMCs) isolated from peptide-immunized mice and splenocytes loaded with no peptide or peptide served as effector cells and target cells, respectively, Cells were co-incubated at effector to target (E:T) ratios of 10,5,1 for use in LDH cytotoxicity assay. The percent lysis of target cells by SMCs were presented as mean ± SD ($n = 5$). *The significant lysis of target cells.

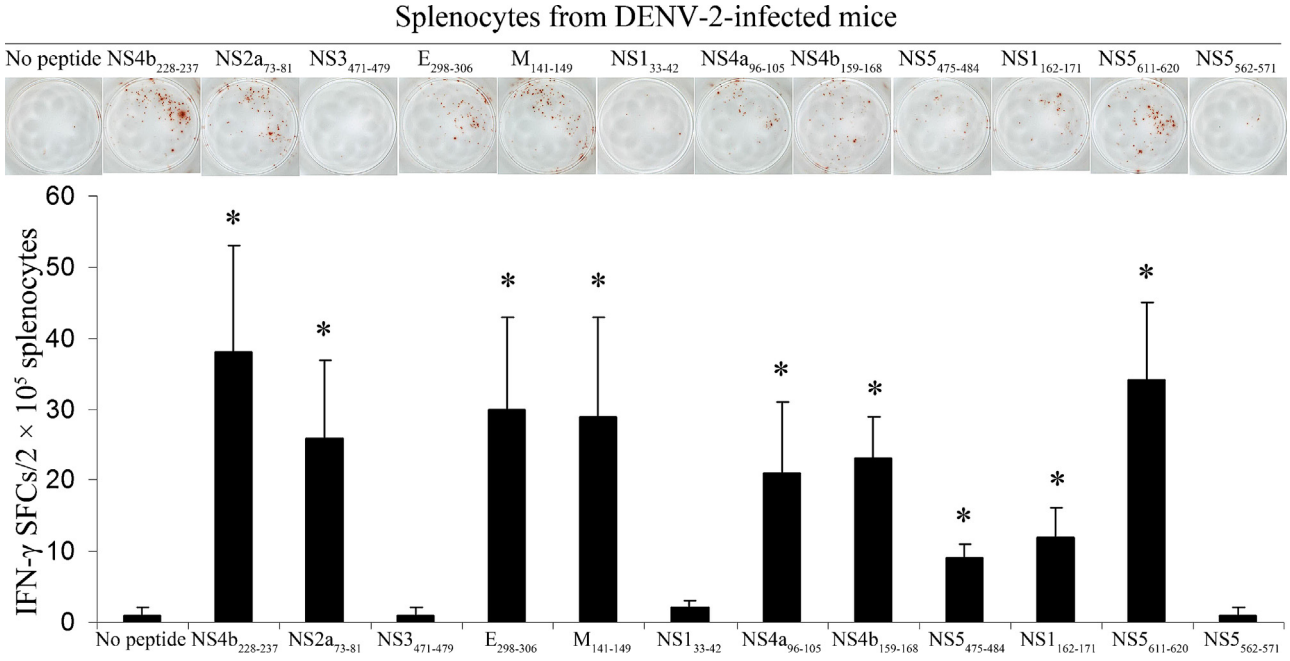

Fig. 4. Peptide-specific IFN-γ-secreting cells in spleen of dengue virus serotype 2-infected HLA-A*2402 transgenic mice. Splenocytes were isolated from DENV-2-infected HLA-A*2402 transgenic mice and were restimulated in vitro with no peptide or individual peptide. The frequencies of IFN-γ SFCs/2 × 10^5 splenocytes were detected using ELISPOT assay and presented as mean ± SD ($n = 5$). *The positive response to a peptide.

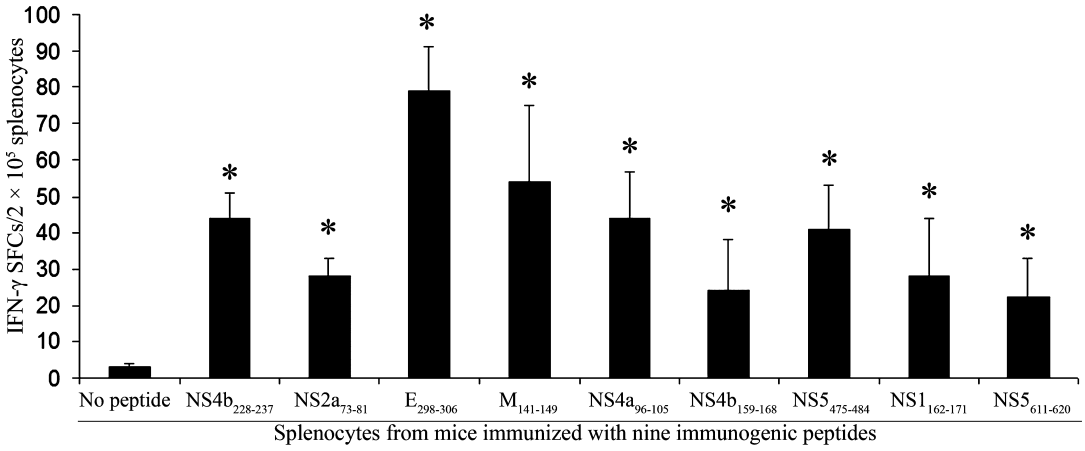

Fig. 5. Peptide-specific IFN-γ-producing cells in spleen From HLA-A*2402 transgenic mice immunized with multiple immunogenic peptides. Splenocytes were isolated from mice immunized with nine immunogenic peptides and were restimulated in vitro with no peptide or individual peptides. The frequencies of IFN-γ SFCs/2 × 10^5 splenocytes were detected using ELISPOT assay and presented as mean ± SD ($n = 5$). *The positive response to a peptide.

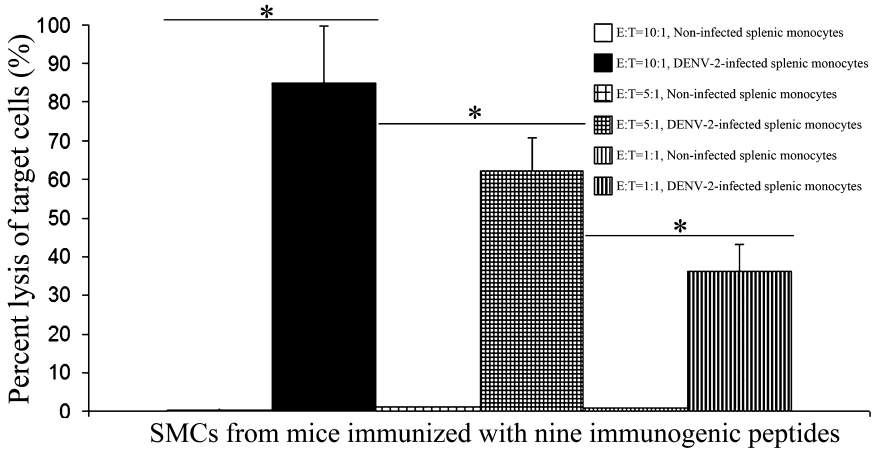

Fig. 6. The cytotoxic activity of splenic mononuclear cells from HLA-A*2402 transgenic mice immunized with multiple immunogenic peptides. Splenic mononuclear cells (SMCs) isolated from mice immunized with nine immunogenic peptides served as effector cells. Both non-infected splenic monocytes and DENV-2-infected splenic monocytes worked as target cells. Cells were co-incubated at effector to target (E:T) ratios of 10,5,1 for use in LDH cytotoxicity assay. The percent lysis of target cells by SMCs were presented as mean ± SD ($n = 5$). *The significant lysis of target cells.

and IL-6 can be produced by DENV-specific $CD8^+$ T cells (Imrie et al., 2007). Peptide-specific T cells were next analyzed to determine if peptide immunization could induce TNF-α and IL-6 production. For 9 immunogenic peptides, $NS4b_{228-237}$-, $NS2a_{73-81}$- and $E_{298-306}$-specific $CD8^+$ cells made both TNF-α and IL-6, whereas $M_{141-149}$ and $NS5_{475-484}$ only induced IL-6 production. Finally, the possible association between peptide-specific T cells and cytotoxicity activity was investigated. It was found that T cells induced by each of 9 immunogenic peptides could lyse peptide-pulsed splenocytes, and the killing rate is significantly correlated with the E: T ratio. The killing activity of peptide-specific T cells at 10:1 ratio of effector cells to target cells ranged from 14.01% to 23.26% (E:T ratio = 10:1). At present, the precise roles of proinflammatory cytokines (especially TNF-α and IL-6) in DENV infection are uncertain. Some studies showed that TNF-α and IL-6 were elevated in patients with DHF when compared to DF (Hober et al., 1993; Green et al., 1999; Chen et al., 2006; Bozza et al., 2008). However, Chen et al. found that IL-6 was elevated in those patients who died due to dengue infections, whereas there was no difference in levels of TNF-α (Chen et al., 2006). Moreover, a larger study carried out by Priyadarshani et al. also showed no difference in serum TNF-α in patients with DHF when compared to those with DF (Priyadarshini et al., 2010). A recent research study suggested that multifunctional $CD8^+$ T cells are associated with protection in DENV infection (Weiskopf et al., 2013). Given that $NS4b_{228-237}$, $NS2a_{73-81}$, $E_{298-306}$, $M_{141-149}$, and $NS5_{475-484}$ could trigger multifunctional T cells, which produce IFN-γ, TNF-α, and/or IL-6 simultaneously, further studies of these peptides may help us understand the exact roles of multifunctional $CD8^+$ T cells in DENV infection.

To further evaluate whether DENV-derived proteins could be processed in mouse cells, DENV-2 was used to infect HLA-A*2402 transgenic mice, and ELISPOT assay was used to detect the ability of splenocytes to recognize individual peptides. The results showed that there are a number of peptide-specific IFN-γ-secreting T cells in DENV-2-infected transgenic mice. Consistent with data in peptide-immunized mice, all 9 of the immunogenic peptides described above were recognized by splenocytes from DENV-2-infected HLA-A*2402 transgenic mice. This means these nine peptides should result from the natural processing of DENV proteins in mouse cells. However, the frequency of peptide-specific IFN-γ-secreting T cells in DENV-2-infected mice are relatively lower than that in peptide-immunized mice. Other studies have confirmed that DENV could infect mouse cells, including macrophages, dendritic cells (Kyle et al., 2007), and splenic macrophages (Prestwood et al., 2012). One recent study showed that DENV can infect HLA transgenic mice and induce the HLA-restricted T-cell response (Weiskopf et al., 2011). In fact, in the present study, DENV-2 was used to infect splenic monocytes, and indirect fluorescence assay was used to detect NS1 on the cell surface before infecting HLA-A*2402 transgenic mice with DENV-2. As a result, most splenic monocytes supported DENV-2 replication (data not shown). In order to determine whether these immunogenic peptides would interfere with each other when used for immunization, the nine immunogenic peptides were mixed together and used to immunize HLA-A*2402 transgenic mice, and the frequency of peptide-specific T cells was evaluated by ELISPOT assay. In line with results in mice immunized with single peptide, IFN-γ-secreting T cells specific for each of the nine immunogenic peptides were detected. Moreover, the SMCs of HLA-A*2402 transgenic mice immunized with 9 immunogenic mice displayed cytotoxic activity against DENV-2-infected splenic monocytes. As expected, the splenocytes from mock-immunized mice did not kill DENV-2-infected splenic monocytes (data not shown). Although the present identified epitopes induced high levels of IFN-γ-secreting T cells, which displayed cytotoxic activity against peptide-pulsed splenocytes and DENV-2-infected splenic monocytes, further research is needed to address whether the peptide immunization could protect animals against DENV attack.

To date, although a lot of DENV-specific T-cell epitopes have been identified, the number of HLA-A*2402-restricted epitopes is limited (Simmons et al., 2005; Lund et al., 2011; Rivino et al., 2012; Nascimento et al., 2013). By comparison, we determined that some of our identified epitopes are similar to previously identified epitopes. For example, Lund et al. reported a DENV-2-specific HLA-A*2402-restricted epitope ($NS5_{475-483}$WYMWLGARF) (Lund et al., 2011). By comparison, we found that the present identified epitope $NS5_{475-484}$WYMWLGARFL has one more residue in the C terminus. In addition, Lund et al. also found a DENV-1-specific HLA-A*2402-restricted epitopes ($NS2a_{73-81}$TYLALMATF). Obviously, the present identified epitope $NS2a_{73-81}$TYLALLAAF is its variant in DENV-2. Nascimento et al. described a HLA-A*2402-restricted epitope D3V-$E_{298-312}$SYAMCTNTFVLKKEV (Nascimento et al., 2013). It is clear that the variant of SYAMCTNTF in DENV-2 is the present identified epitope $E_{298-306}$SYSMCTGKF. Although Rivino et al. indicated that DENV-2-$NS1_{161-175}$GVFTTNIWLKLKEKQ and DENV-2-$NS5_{561-575}$HKKLAEAIFKLTYQN are novel $CD8^+$ T-cell epitopes, they did not determine the HLA restriction of these epitopes (Rivino et al., 2012). Compared with our epitopes, we were

able to determine that DENV-2-NS1$_{161–175}$GVFTTNIWLKLKEKQ and DENV-2-NS5$_{561–575}$HKKLAEAIFKLTYQN contain the sequences of NS1$_{162–171}$VFTTNIWLKL and NS5$_{562–571}$KKLAEAIFKL, respectively.

In conclusion, we identified nine immunogenic HLA-A*2402-restricted epitopes that are highly conserved in hundreds of DENV-2 isolates. Among these immunogenic peptides, five peptides are similar to previously identified epitopes and the remaining four peptides are novel epitopes. This study contributes to the understanding of CD8$^+$ T-cell response following DENV infections and provides useful information for future vaccine development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2014.10.022.

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