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VP2 Dominated CD4⁺ T Cell Responses against Enterovirus 71 and Cross-Reactivity against Coxsackievirus A16 and Polioviruses in a Healthy Population

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Enterovirus 71 (EV71)–associated hand-foot-mouth disease has become a major threat to public health in the Asia–Pacific region. Although T cell immunity is closely correlated with clinical outcomes of EV71 infection, little is known about T cell immunity baseline against EV71 and T cell immunogenicity of EV71 Ags in the population, which has restricted our understanding of immunoprotection mechanisms. In this study, we investigated the cellular immune responses against the four structural Ags of EV71 and determined the immunohierarchy of these Ags in healthy adults. A low frequency of EV71-responsive T cells was detected circulating in peripheral blood, and broad T cell immune responses could be identified in most of the subjects after in vitro expansion. We demonstrated that the VP2 Ag with broad distribution of immunogenic peptides dominates T cell responses against EV71 compared with VP1, VP3, and VP4. Furthermore, the responses were illuminated to be mainly single IFN-γ-secreting CD4⁺ T cell dependent, indicating the previous natural acute viral infection of the adult population. Conservancy analysis of the immunogenic peptides revealed that moderately variant peptides were in the majority in coxsackievirus A16 (CV-A16) whereas most of the peptides were highly variant in polioviruses. Less efficient cross-reactivity against CV-A16 might broadly exist among individuals, whereas influences derived from poliovirus vaccination would be limited. Our findings suggest that the significance of VP2 Ag should be addressed in the future EV71-responsive immunological investigations. And the findings concerning the less efficient cross-reactivity against CV-A16 and limited influences from poliovirus vaccination in EV71-contacted population would contribute to a better understanding of immunoprotection mechanisms against enteroviruses. The Journal of Immunology, 2013, 191: 1637–1647.

Since its first identification in California in 1969, enterovirus 71 (EV71)–associated hand-foot-mouth disease (HFMD) epidemics have been reported throughout the world, with large outbreaks mainly occurring in the Asia–Pacific region since 1990s (1–7). Although EV71 and coxsackievirus A16 (CV-A16) are the most common causative agents of HFMD, infection by EV71 usually carries a higher risk of developing central nervous complications and fatalities (8). In 2008, a nationwide EV71 outbreak started in Fuyang City, China, has caused ~5000 cases and >188 deaths in mainland China (9). During the following years, >1 million incidences and hundreds of deaths have been reported annually in China, and EV71 has become a major threat to public health (http://www.moh.gov.cn/publicfiles/business/htmlfiles/wsb/pyqxx/list.htm). In the first half of 2012, the so-called “mystery disease” in children that caused >50 deaths in Cambodia was also found to be EV71-associated HFMD (10). A continued lack of therapeutic medication and an effective vaccine for EV71 will likely lead to persistent periodical epidemics and dissemination in the near future (11). Thus, effective vaccines that would prevent the prevalence of EV71 infection in at-risk populations are in urgent need for the control of HFMD around the world. Nevertheless, the mechanisms of EV71 pathogenesis remain unclear, and child-orientated epidemics and severe neurologic complications with unknown causes have made the study of the immunogenicity of EV71 Ags and the roles they play in the protection against infection of significant importance.

As a major member of the Enterovirus genus of the family Picornaviridae, EV71 is a nonenveloped ssRNA virus coated with an icosahedral capsid containing 60 copies of four structural proteins, VP1, VP2, VP3, and the inner VP4 (12). The four structural proteins are the major Ags of EV71 and have thus been the focus of vaccine development and applied as the main effective constituents in vaccine effectiveness evaluations (13, 14). In particular, VP1 is highly exposed, as well as considered the main target of neutralizing Abs, and has thus been broadly used for subunit vaccine development (15–17). The lower prevalence of neutralizing Abs accompanied by higher transmission rates in children and infants indicates that neutralizing Abs are important for the prevention of
EV71 infection (8, 18). However, 80% of EV71-infected patients become positive for EV71-specific neutralizing Abs 1 d after the onset of illness, and the magnitude of Ab responses is not correlated with disease severity and outcome (19). In contrast, documented clues imply that cellular but not humoral immune responses are correlated with disease progression and clinical outcome (20, 21).

Current studies on host immune responses against EV71 indicate that T cell immunity plays a critical role in protection against EV71 infection and control of the disease (20, 22). Previous work demonstrates that decreased cellular immunity and lower IFN-γ and other cytokine/chemokine responses are correlated with more severe clinical outcome of EV71 infection, whereas the neutralizing Ab titers display no difference between mild, severe, and even fatal cases (22). Altered cellular immunity associated with polymorphisms of CTLA-4 is suggested to be correlated with disease severity (20). Likewise, studies in animal models reveal that cellular immune-related lymphocytes or cytokines reduce the lethality of EV71-infected mice by decreasing viral loads in tissues (23–26). These observations lead to the hypothesis that T cells might be crucial in protection and immunity against EV71.

Despite its importance, our knowledge of human cellular immunity toward EV71 is still limited. To date, only a few studies have assessed cellular immunity against EV71 in small cohorts of subjects based on in silico predicted peptides (27–29). Thus, the magnitude of human T cell responses to EV71 and the immunohierarchy of EV71 Ags remain unclear. Furthermore, the phenotype and functionalities of EV71-responsive T cells in humans remain to be elucidated. A better understanding of all of these aspects would be highly valuable to vaccine development and understanding EV71 pathogenesis.

To investigate human cellular immunity to EV71 and the immunohierarchy of the four structural proteins of EV71, we synthesized overlapping peptides spanning all the four structural proteins (VP1–VP4) and performed a study of viral Ag-specific T cell responses in adults. In addition, we also determined the phenotype and functional features of dominant Ag-specific T cells and their cross-reactivity with different genotypes of EV71, CV-A16, and polioviruses at the individual peptide level. These data will aid in the understanding of host protection mechanisms against EV71 and effective vaccine development.

Materials and Methods

Study subjects

Thirty healthy subjects (age, 20–51 y) from Beijing, China, were recruited in this study. All of the subjects displayed no symptoms of HFMD and had no history of EV71 infection or close contact with EV71-infected individuals during the sampling period. Written informed consent was obtained from all of the subjects, and the study was approved by the Ethics Review Committee of the Institute of Microbiology, Chinese Academy of Sciences. The study was conducted in accordance with the principles of the Declaration of Helsinki, the standards of Good Clinical Practice (as defined by the International Conference on Harmonization).

Lymphocyte purification and in vitro generation of polyclonal T cell lines

PBMCs were isolated from the whole blood of donors by density gradient centrifugation using Ficoll–Hypaque (TBD Science) and washed twice in RPMI 1640 medium containing 10% FBS (Life Technologies) (30). Freshly isolated PBMCs in RPMI 1640 medium supplemented with 10% FBS were incubated with peptide pool (with final concentration of 5 μg/ml for each peptide) at 37°C in 5% CO2 at a density of 2 × 10^6 cells/ml in a 24-well culture plate. rIL-2 (20 IU/ml) was added to the culture medium on day 3. Half of the medium was replaced with fresh medium supplemented with 20 IU/ml rIL-2 on day 7. Peptide-specific T cells were tested via ELISPOT assays on day 10.

Neutralizing Ab detection

Neutralizing Ab against EV71 and CV-A16 were detected, respectively, with microneutralization test on human rhabdomyosarcoma cell on 96-well flat-bottom cell culture microtiter plate as described previously (31). Sera were incubated at 56°C for 30 min at first and prepared by 4-fold serial dilutions ranging from 1:8 to 1:256. Serum samples (50 μl) were added to each well with duplicated wells for each dilution. Then, 50 μl virus (EV71 or CV-A16) with 100% 50% cell culture infective dose were distributed to each well. The EV71 isolate (subgenotype C4a; GenBank accession number EU703812) and the CV-A16 isolate (subgenotype B1b; GenBank accession number GQ429229) were used in the analysis. After thorough mixture, virus and serum were incubated at 36°C for 2 h. After that, the virus would be neutralized and lose infectivity to cells if specific Ab exists in serum. Then, 100 μl cell suspension containing 2 × 10^5/ml rhabdomyosarcoma cells was added to each well, and the plate was cultured in CO2 incubator at 36°C for 7 d. Cytopathogenic effect was examined on day 7, and the dilution of serum, which could protect 50% cell from appearance of cytopathogenic effect, was designated as the titer of the neutralizing Ab to specific virus. Serum control was set for each specimen to observe any nonspecific toxic effect to cell at 1:8 dilution, with virus replaced with cell maintain medium. To make sure the virus used to attack was infective, virus control was set for each plate, with serum replaced with maintain medium. The method was previously calibrated using reference sera of EV71 and CV-A16, and no cross-reaction was found (31). Finally, neutralizing titration ≥ 1:8 was considered to be positive (9, 31, 32).

Synthetic peptides and peptide matrix pool design

To evaluate T cell responses against EV71, each of the four structural proteins of EV71 from the 2010 Henan strain (GenBank accession number ADX87405.1) was selected as a template sequence for overlapping peptide design. A total of 110 15- to 19-mer C-terminal–adapted peptides overlapping by 10 aa and spanning all of the structural proteins (VP1, VP2, VP3, and VP4) were designed and synthesized (purity > 90%; China-Peptide) as described previously (Supplemental Table I). The peptides from VP1–VP4 were further assigned to two pools according to the sequence position (designated as the N- and C-terminal pools) for each Ag to minimum the matrix pools needed to screen the positive peptides in the pool (Table I).

ELISPOT assay

The Ag-specific T cell responses were detected with an IFN-γ-secreting ELISPOT assay (Quantobio) (34, 35). A total of 2.5 × 10^5 freshly isolated PBMCs from donors in 100 μl RPMI 1640 medium supplemented with 10% FBS were seeded in each well of 96-well ELISPOT plate membranes precoated with 10 μg/ml anti-INF-γ mAb. To detect in vitro–cultured T cell lines, 5 × 10^5 cells were added to each well. To stimulate the effector cells, individual peptide (with final concentration of 10 μg/ml) or peptide pools (with final concentration of 5 μg/ml) for each peptide diluted in 100 μl RPMI 1640 medium supplemented with 10% FBS were added to each well and incubated at 37°C with 5% CO2 for 18 h. PHA (36) was added as a positive control for nonspecific stimulation. Cells incubated without stimulator were employed as a negative control, which produced less than five spots in 90% of the experiments. All of the wells were duplicated to minimize possible discrepancies. Then, the cells were removed, and the plates were processed according to the manufacturer’s instructions. Finally, the colored spots, which represent epitope-specific T cells, were counted and analyzed using an automatic ELISPOT reader (CTL). Responses were considered to be positive when the spots-forming cells (SFCs) in the target well were more than five spots and greater than two times the average negative control value. The adjusted average SFCs after subtracting the mean negative control values are presented as SFCs per 10^4 or 10^5 PBMCs in the determination of freshly isolated PBMCs or in vitro–expanded T cell lines, respectively.

CD4+ and CD8+ T cell depletion

 Initially confirmed positive Ag pools or peptide-specific responses were subsequently characterized by CD4 or CD8 depletion of expanded T cell lines using anti–CD4+ or anti–CD8-coated magnetic beads (MACS) (37). Briefly, polyclonal T cell lines generated by expansion in the presence of corresponding peptide pools or peptide were incubated with anti–CD4+ or anti–CD8-coated magnetic beads for 15 min at 4°C. Then, the mixture was washed and resuspended in PBS containing 0.5% FBS and 2 mM EDTA-Na and passed through a strong magnetic field for depletion of CD4 or CD8 populations. The CD4- or CD8-depleted T cell lines were collected.
for ELISPOT assays to analyze the CD4 and CD8 distribution of the peptide pool or peptide-specific responses.

**Intracellular cytokine staining and flow cytometry**

After in vitro culture, T cell lines were rested for 2 h and then stimulated with a specific peptide pool (5 μg/ml for each peptide) or peptide (10 μg/ml) for 2 h and incubated for an additional 4 h with GolgiStop/monesin (BD Biosciences) at 37°C in 5% CO2 (30). Cells cultured with medium alone or PHA were used as negative and positive controls, respectively. To determine CD4 and CD8 phenotypes, the cells were harvested and stained with anti-CD4 allophycocyanin and/or anti-CD8 FITC surface markers, fixed, and permeabilized in permeabilizing buffer (BD Biosciences), and stained with anti–IFN-γ PE-Cy7 (BD Biosciences). To analyze the functional properties of the peptide-specific peptides, T cell lines were stained with anti-CD4 FITC as a surface marker and then intracellularly stained with anti–IFN-γ PE-Cy7 and anti–IL-2 allophycocyanin. All fluorescent lymphocytes were gated on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with FlowJo software.

**Statistical analysis**

Differences in mean values were evaluated for statistical significance (p < 0.05 or < 0.01) by the Student two-tailed t test. Data were assembled and statistically calculated using Excel (Microsoft) or Origin 8.0 (OriginLab).

**Results**

**Overall humoral and cellular immunohierarchy in the peripheral blood of healthy donors against EV71 and CV-A16**

Blood samples from 30 healthy adults were collected to evaluate their humoral and cellular immune responses against EV71 (male: female = 19:11, age 28 ± 6.1 y). Humoral immune responses were evaluated by determining neutralizing Ab titers against EV71 and CV-A16. Generally, EV71-dominated neutralizing Ab responses were demonstrated among the study subjects with titers of ≥1:8 and showed an overall positive rate of 80% (24 of 30) (Fig. 1A).

In contrast, less than half of the subjects (47%; 14 of 30) were positive for CV-A16–specific neutralizing Abs. A significantly higher responsive magnitudes were also demonstrated against EV71 (average of 1:36) than CV-A16 (average of 1:9) among the subjects (p < 0.001). Specifically, EV71-only responses were detected in 41.7% (10 of 24) of the subjects who possess undetectable CV-A16 humoral responses, whereas superior (5 of 24) or equal (9 of 24) responses against EV71, compared with CV-A16, were detected among the other subjects. In contrast, no CV-A16-only or CV-A16–superior responses were detected among the study subjects. These findings are in accordance with the EV71-dominated epidemics in China (31, 38, 39). EV71-dominated neutralizing Ab responses among the study subjects suggested a previous contact of EV71 virus in most of the study subjects and ensured that the investigation of EV71 responsive cellular immunity among these subjects was reasonable and feasible.

To evaluate T cell responses against EV71 in a healthy population, we synthesized overlapping peptides spanning the entire sequence of the four structural proteins of EV71. A more conserved EV71/Henan/DC/2010 strain was selected as the template strain for overlapping peptide design. As a result, 110 C-terminal–adapted 15- to 19-mer peptides overlapping by 10 aa were designed and further assigned to seven pools (Table I). Freshly isolated PBMCs from 30 donors were separately challenged with the seven peptide pools, and T cell responses were determined by ex vivo ELISPOT assays and analyzed as SFCs per 106 PBMCs. According to the positive criteria (see Materials and Methods), mild T cell responses against EV71 were detected in seven donors (with a mean response of 25.4 ± 4.7 SFCs/106 PBMCs). Interestingly, all seven positive responses were against the VP2 Ag: four to the N-terminal pool and the other three to the C-terminal pool. Among these individuals, four had neutralizing Ab titers ≥1:16 against EV71, but the others had no detectable neutralizing Abs. These results revealed that mild T cell responses against EV71 could be detected in a healthy population with or without the presence of EV71-neutralizing Abs. The magnitude of responses against VP2 were significantly higher than that against the other Ags (p < 0.01), which indicated that VP2 dominated the T cell responses against EV71 (Fig. 1B).

**The dominant role of VP2-specific T cell responses**

To investigate the possible low-frequency T cells against EV71, the remainder of the PBMCs collected from all of the donors were individually cultured in the presence of the seven peptide pools in vitro for 9 d, and T cell responses against EV71 were then evaluated from the expanded T cells. Unexpectedly, on the basis of the criteria, we used to score a “positive” response (see Materials and Methods), 28 of the subjects (93%) displayed significant positive responses to at least one of the four structural Ags (Fig. 2A, 2B). Similar to the ex vivo ELISPOT results, dominant responses were
VP2 specific, and positive responses against VP2 were detected in 26 subjects (i.e., 87% of the study population (Fig. 2B)). More specifically, 57% (17 of 30) and 67% (20 of 30) of the responses were specific to the VP2-N- or -C-terminal peptide pools, respectively. In contrast, the recognition frequency for VP1 (70%) and VP3 (77%) was much lower, with the lowest recognition rate for VP4 (47%) (Fig. 2B). Furthermore, we analyzed the correlation between the magnitudes of EV71 or CV-A16–neutralizing Ab titers with T cell responsive magnitudes against the four Ags. No significant correlation was observed in any of the Ag or overall responses as a whole with neutralizing Ab titers against EV71 or CV-A16 (p > 0.05).

Next, we analyzed the immunohierarchy of the four structural Ags in the subjects. The level of responses from in vitro–expanded PBMCs was both related to the proliferation rate of Ag-specific T cells in different individuals and determined by the memory precursors and phenotypes of the virus-specific T cells in different donors. From the analysis, we observed that the VP2 Ag elicited a mean of 171 SFCs/10^5 PBMCs among the 28 subjects positive against EV71, which was much higher compared with 73, 67, and 44 SFCs/10^5 PBMCs for the VP1, VP3, and VP4 Ags, respectively (p < 0.01) (Fig. 2C). Moreover, the magnitudes of the VP2-specific responses among different individuals were well correlated with the overall T cell responses to the four structural Ags as a whole (r = 0.84; p < 0.01) (Fig. 2D). Taken together, these findings demonstrated that the VP2 Ag dominates T cell responses against EV71 in an adult population.

**Uncommon diverse distribution of T cell immunogenic regions in VP2 protein**

We next sought to determine the shorter immunodominant regions toward which the immunogenicity of the four structural Ags was specified. In this section, only subjects who displayed a positive

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**Table I. Summary of designed overlapping peptide pools spanning the four structural Ags of EV71**

<table>
<thead>
<tr>
<th>Ag</th>
<th>Length (amino acids)</th>
<th>Peptide Pool</th>
<th>Amino Acids Spectrum</th>
<th>No. of Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1</td>
<td>297</td>
<td>VP1-N-pool</td>
<td>1–162</td>
<td>20</td>
</tr>
<tr>
<td>VP1</td>
<td>254</td>
<td>VP1-C-pool</td>
<td>145–297</td>
<td>19</td>
</tr>
<tr>
<td>VP2</td>
<td>242</td>
<td>VP2-N-pool</td>
<td>1–126</td>
<td>16</td>
</tr>
<tr>
<td>VP2</td>
<td>254</td>
<td>VP2-C-pool</td>
<td>111–241</td>
<td>16</td>
</tr>
<tr>
<td>VP3</td>
<td>242</td>
<td>VP3-N-pool</td>
<td>1–132</td>
<td>15</td>
</tr>
<tr>
<td>VP3</td>
<td>254</td>
<td>VP3-C-pool</td>
<td>115–254</td>
<td>15</td>
</tr>
<tr>
<td>VP4</td>
<td>69</td>
<td>VP4</td>
<td>1–69</td>
<td>8</td>
</tr>
</tbody>
</table>

*Overlapping peptides were designed using EV71/HENAN/DC/2010 (GenBank accession number ADX87405.1) strain as template.

*Amino acids spectrum of each peptide pool was presented from N- to C-terminal of each Ag.*

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**FIGURE 2.** Immunohierarchy of four EV71 structural Ags and the dominance of the VP2 Ag. (A) All participants are represented on the x-axis, and the total magnitudes of the IFN-γ ELISPOT responses against the four Ags are represented on the y-axis. The results represent the average SFCs per 10^5 PBMCs from two duplicate wells, after subtracting the mean negative control values in the individual. Each colored segment represents the source Ag of the corresponding responses. (B) Differential recognition of four Ags among the individuals. According to the positive criterion for ELISPOT analysis of in vitro–expanded PBMCs, the overall recognition frequency of the four Ags in the study cohort is presented on the y-axis. The shadow on each colored bar represents the frequency of dual recognition to the N and C terminus of the corresponding Ag. The upper parts represent the recognition of the N-terminal peptide pool, and the lower parts represent the recognition of the C-terminal peptide pool of a given Ag. Overall recognition frequency was designated as "EV71." (C) Magnitudes of the average responses to the four Ags among the 28 individuals who showed positive responses. The SE was represented on each Ag bar. **p < 0.01. (D) The magnitude of VP2-specific responses was highly correlated with the overall responses to the four Ags as a whole (r = 0.84, p < 0.01).
response to a particular peptide pool were involved to determine more specific responses. Our results demonstrated that the responses to VP1, VP3, and VP4 were mainly focused on the central region of the Ag, although the C terminus of VP1 was also recognized in some of the subjects (Fig. 3A, 3C, 3D). Interestingly, the responses against VP4 among all of the responsive subjects were highly focused on two overlapped peptides in a 23-aa central region (VP4 (18-41)). Unlike the other three Ags, responses to VP2 were widely distributed from the N- to the C terminus of the protein in the study subjects (Fig. 3B).

Next, two-dimensional matrix peptide pools were designed to determine the particular individual peptides responsible for the positive responses in the pools, and promiscuous peptides were further determined by repeated ELISPOT assays using individual peptides with the same T cell line. As a whole, 31 peptides containing T cell epitopes were identified in the four structural Ags, including two HLA-DR–restricted epitopes previously identified by other groups (Table II) (27). The most abundant peptides (13 peptides) were defined within the VP2 Ag, indicating that VP2 had the highest epitope density (5.4 responsive peptides per 100 aa) compared with VP1, VP3, and VP4 (3, 2.8, and 2.9 responsive peptides per 100 aa, respectively). Distribution of the immunogenic peptides on the four structural Ags was presented together with the identified B cell epitopes from previous literatures (Supplemental Fig. 1). Given all of the above data, the uncommon diversely distributed T cell responses spanning the entire Ag in donor 1 were further analyzed with intracellular cytokine staining (ICS) assays by flow cytometry. ICS assays further confirmed that T cell responses to VP2 were mainly CD4+ T cell dependent. VP2-specific CD4+ T cells accounted for 95% of the IFN-γ–secreting cells, whereas only 5% were CD8+ dependent (Fig. 4C). In contrast, the VP2–26 (192–209) peptide-specific T cells were completely CD4+ T cell dependent. In addition, two major CD4+ T cell functional cytokines, IFN-γ and IL-2, were analyzed to characterize the functional properties of CD4+ T cells at the individual peptide level. The results demonstrated that the VP2–26 (192–209) peptide-specific T cells were mainly single IFN-γ–producing T cells (5.05% of the total CD4+ T cells), with a low level of polyfunctional T cells simultaneously secreting IFN-γ and IL-2 (0.14% of the total CD4+ T cells) (Fig. 4D).

**FIGURE 3.** Diverse distribution of VP2-specific T cell immunogenic regions. The T cell responses were specified to a region of 40–50 aa for VP1 (A), VP2 (B), VP3 (C), and VP4 (D). Four or five peptides were mixed together to constitute a peptide pool, which represented the responses of a specific region of the Ag. The magnitude of responses is represented as SFCs per 10^6 PBMCs on the y-axis, whereas the peptide spanning region and subjects are represented on the x- and z-axes, respectively. Unlike the highly focused distribution of responses to the central region in VP1, VP3, and VP4, the responses to VP2 were more diversely distributed along the Ag (B).

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**LESS EFFICIENT CROSS-REACTIVITY AGAINST MODERATELY VARIANT PEPTIDES FROM CV-A16**

EV71 is most genetically related to CV-A16, which is another major causative pathogen of HFMD; the amino acid identity of all of the structural Ags is ~78% between the two viruses (40). Moreover, EV71 comprises three major genotypes, and each genotype contains various subgenogroups circulating in different regions around the world (41). Thus, we performed conservancy analysis of the identified peptides between EV71 and CV-A16 viruses and among different EV71 genotypes. Of the 31 confirmed peptides containing EV71 T cell epitope regions, 29 displayed varied amino acid substitutions compared with CV-A16 (with an average variation of 2.8 aa per peptide) (Table II). We
The presence of high peptide concentrations. Besides, EC50 also was calculated to determine the sensitivity of cross-reactivity against variant peptides. Cross-reactivity against the variant peptides could be detected though the responses differed among different subjects (Fig. 5B). Moderate to strong cross-reactivity against variant VP2–26-EV71A/B and VP2–26-CV16 was detected in donor 1 and donor 25 who showed robust responses against VP2–26-EV71C, whereas no cross-reactivity was detected in donor 5 who showed weak responses against VP2–26-EV71C. EC50 of the responses against VP2–26-EV71C (0.038 μg/ml) in donor 1 was lower than VP2–26-EV71B (0.0608 μg/ml), whereas highest EC50 (0.127μg/ml) was detected against VP2–26-CV16, which indicated the less efficient cross-priming of T cell responses by the variant peptide from CV-A16. Responses in donor 25 showed similar lower EC50 (0.065 μg/ml) against VP2–26-EV71C than VP2–26-CV16 (0.214 μg/ml). Although neutralizing Ab titers specific to EV71 and CV-A16 were both detected in these donors, our findings implied that T cells existed in the peripheral blood were likely elicited from natural infection by EV71 genotype C strains, which is in accordance with the EV71 C4-dominated epidemics in mainland China (38, 41). All these results indicated that cross-reactivities against the moderately variant peptides from CV-A16 broadly existed among different subjects, although they may be less efficient in priming T cell responses than the variant peptides derived from EV71.

Limited influences to EV71-responsive cellular immunity derived from vaccination of polioviruses

Poliovirus was another member of Enterovirus genus and poliovirus vaccine strains shared ~42% identity in capsid Ags with that of EV71. Programmed vaccination of poliovirus vaccines was carried out national wide among children in China, and most of the subjects enrolled in this study claimed to have received poliovirus vaccination previously. Thus, we next sought to investigate the

Table II. Summary of the identified immunogenic peptides

<table>
<thead>
<tr>
<th>Source Ag</th>
<th>Peptides</th>
<th>Amino Acid Location</th>
<th>EV71</th>
<th>CV-A16 Equivalent Region</th>
<th>Recognition Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1 9</td>
<td>VP1–1(75–90)</td>
<td>TATLTDSSFSRAGLTV</td>
<td>−Q−AG−</td>
<td>1/7</td>
<td></td>
</tr>
<tr>
<td>VP1–16(112–129)</td>
<td>DISGTAQRRRVRVEFYTMY</td>
<td>−L−</td>
<td>4/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1–17(120–137)</td>
<td>RVRVLEMTFMDAMFTTV</td>
<td>−C−</td>
<td>2/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1–19(137–154)</td>
<td>RVRVEFMDTGFDVNLLOQ</td>
<td>−V−AK−N−</td>
<td>1/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1–20(145–162)</td>
<td>EVPGQLQYVFVPGAPK</td>
<td>−L−Y−</td>
<td>1/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1–23(169–187)</td>
<td>ARQTAStNPVFSKVLQQPA</td>
<td>−I−T−</td>
<td>1/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1–33(244–261)</td>
<td>KYPDVLVTRMRKHRAW</td>
<td>−PHSITL−V−I−</td>
<td>2/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1–34(252–267)</td>
<td>YMRHVRAWITPRMR</td>
<td>−I−I−</td>
<td>1/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1–37(271–285)</td>
<td>YLFLQANYYAGESTR</td>
<td>−K−D−</td>
<td>2/5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP1–39(287–301)</td>
<td>AGQVTGSNTTVGAAANI</td>
<td>−Q−</td>
<td>3/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP2 13</td>
<td>VP2–5(29–47)</td>
<td>ANTVAGDEMPVCYSDDDA</td>
<td>−IA−</td>
<td>E−K−A−</td>
<td></td>
</tr>
<tr>
<td>VP2–9(62–79)</td>
<td>RFTTVLTDNLEDSSKGY</td>
<td>−S−A−</td>
<td>2/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP2–10(70–86)</td>
<td>LDEESKKWVFSVPDL</td>
<td>−S−A−</td>
<td>2/7</td>
<td></td>
<td></td>
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<tr>
<td>VP2–11(77–92)</td>
<td>GNYLRFDPVLTTSFV</td>
<td>−V−</td>
<td>1/7</td>
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<tr>
<td>VP2–12(83–100)</td>
<td>PTDVLPDDGQGNAQHY</td>
<td>−V−</td>
<td>2/7</td>
<td></td>
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<tr>
<td>VP2–15(107–224)</td>
<td>CIIVCHASKFKHQQALLV</td>
<td>−V−</td>
<td>1/7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VP2–16(115–132)</td>
<td>SEKPHGGALLVYMLPEVY</td>
<td>−I−L−</td>
<td>2/7</td>
<td></td>
<td></td>
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<tr>
<td>VP2–19(139–155)</td>
<td>TQEDETHPKVDGOPA</td>
<td>−D−N−S−</td>
<td>VT−Q−</td>
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<tr>
<td>VP2–21(152–169)</td>
<td>PGSSDFQLOHPVLDDAG</td>
<td>−N−</td>
<td>1/6</td>
<td></td>
<td></td>
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<tr>
<td>VP2–24(176–193)</td>
<td>TVCPIHQWNLRTNNTCATI</td>
<td>−</td>
<td>3/6</td>
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<tr>
<td>VP2–25(184–201)</td>
<td>NLRTNNCATIVPYIVAL</td>
<td>−</td>
<td>1/6</td>
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<tr>
<td>VP2–26(192–209)</td>
<td>TIIVPYNALPPSDALN</td>
<td>−M−TV−</td>
<td>3/6</td>
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<tr>
<td>VP3 7</td>
<td>VP3–5(50–61)</td>
<td>HELLILQQVTLIEEYIV</td>
<td>−R−B−</td>
<td>1/2</td>
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<tr>
<td>VP3–7(49–66)</td>
<td>VETILEVNPYNVSNL</td>
<td>−IQS−E−TP−</td>
<td>1/2</td>
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<tr>
<td>VP3–14(99–115)</td>
<td>TMLGQCGYTYQWGL</td>
<td>−R−</td>
<td>1/2</td>
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<tr>
<td>VP3–17(118–135)</td>
<td>TFFMTSGDAMTKGKLIA</td>
<td>−A−I−</td>
<td>1/5</td>
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<tr>
<td>VP3–18(126–143)</td>
<td>MALTGMMIALTPQPGPLE</td>
<td>−I−</td>
<td>5/7</td>
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<tr>
<td>VP3–20(142–169)</td>
<td>LPDKRAMLATGLTHWTVDF</td>
<td>V−A−L−</td>
<td>5/7</td>
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<tr>
<td>VP4 2</td>
<td>VP4–3(34)</td>
<td>SMT2STITYNTTNYKYK</td>
<td>−S−</td>
<td>4/7</td>
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<td>VP4–29(41)</td>
<td>INTTNYKXDSSAATA</td>
<td>−A−S−</td>
<td>7/7</td>
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influence of T cell responses derived from poliovirus vaccination to the EV71-responsive T cells identified in the current study.

We first analyzed variation levels of the corresponding peptides in polioviruses compared with the immunogenic peptides identified from EV71 among the detected subjects. All of the immunogenic peptides identified in EV71 contained more than three amino acid variations in all the three poliovirus vaccine strains, and more than 80% of the peptides were highly varied with 5–16 aa variations and an average of 8.9 aa variations within each peptide (Fig. 6A). Cross-reactivity of EV71-responsive T cells against polioviruses were further determined in three subjects with the representative peptide, VP2–26 (192–209), which had 10 aa variations in polioviruses (VP2–26-Polio, TLVLPYVNSLSIDSMVKH). PBMCs from the subjects were cultured in the presence of VP2–26-EV71C or VP2–26-Polio, separately, and then the expanded polyclonal T cells were detected against VP2–26-EV71C, VP2–26-CV16, and VP2–26-Polio. The results showed that T cells derived from VP2–26-EV71C stimulation could be responsive to variant peptides VP2–26-EV71C and VP2–26-CV16 but failed to response against VP2–26-Polio (Fig. 6B). Besides, no responses against any of the variant peptides could be detected from VP2–26-Polio–stimulated T cells (Fig. 6C). Taken together, T cell responses against EV71 were dominated by immunogenic peptides, which were highly varied from that of polioviruses, and thus, the influences derived from previous poliovirus vaccination to EV71-responsive cellular immunity would be limited.

**FIGURE 4.** Phenotype and functional characterization of VP2-specific T cells. The CD4/CD8 T cell dependency of VP2-specific T cell responses was determined by CD4/CD8-depleted ELISPOT analysis (A, B) and ICS assays (C). The responses of CD4- or CD8-depleted or untreated T cells from donor 1 are designated as D1_CD4, D1_CD8, and D1_PBMC (respectively) and are represented with different shaped bars (A); the responses in donor 5 are likewise represented (B). “M” is the negative control cultured with medium alone, and “PHA” is the positive control cultured with PHA. In ICS analysis with IFN-γ, the phenotypic dependency of VP2-specific responses was further confirmed to be CD4-dominated (upper), although low frequency CD8 T cells could be detected (59), whereas responses to the immunodominant peptide VP2–26 (192–209) were completely CD4+ T cell dependent (C). Functional properties of VP2–26 (192–209)-specific T cells were determined through staining of IFN-γ (y-axis) and IL-2 (x-axis). The T cell populations with different cytokine combination properties were gated on CD4+ T cells and represented in different quadrants on the plot (D). For VP2–26 (192–209) peptide–specific T cells, dominant single IFN-γ–secreting T cells are shown in the upper left quadrant, whereas low-frequency dual cytokine–secreting T cells are shown in the lower right quadrant. Numbers in the quadrants of each plot represent the frequency of positive T cells in each quadrant. Cells cultured with medium alone or PHA were used as negative or positive controls, respectively. These results are representative of three independent experiments.
When the virus persistently circulated in China since 2000 (38).

Previous studies of cellular immunity to enteroviruses mostly pay greater attention to the VP1 Ag (48–50). Nonetheless, a study of the immunohierarchy of conserved peptides from four structural Ags from enteroviruses, including Poliovirus 1, coxsackie B3, B5, B6, and A9, and Echovirus 30, on a small cohort of subjects revealed that cross-reactive T cell epitopes are mainly located in VP2 and VP3 (and to a lesser extent in VP1) (51). For EV71, investigation of T cell immunity has also mainly focused on VP1, and several HLA-class II–restricted T cell epitopes have been determined within this Ag (27, 29). Moreover, a collection of studies demonstrate that VP1 is the dominant target of neutralizing Abs in EV71, and thus, VP1 has been broadly explored for subunit vaccine development, priming strong humoral immunity in a mouse model (15, 52, 53). However, compared with an N-terminal–biased neutralizing determinants distribution in humans, distribution of neutralizing Ab epitopes in mouse model reveals no N- or C-terminal bias, and numbers of epitopes are identified in the C-terminal of VP1 in mouse model (52, 54, 55). Besides, the pathology of EV71 infection in mice and humans is also quite different (56). Thus, the immunity and protection efficiency of subunit vaccines in the mouse model cannot be analogized to that in humans. Thus, Ag candidates for an effective vaccine should be rich in both B and T cell epitopes. Although VP1 is the major target of neutralizing Abs, our present study demonstrated that T cell responses were dominated by the VP2 Ag. Wei et al. (57) also identified a highly conserved dominant T cell epitope in VP2 Ag from an silico–predicted peptide pool which is included in the identified peptides in the current study (VP2–24[176–193] peptide) (Table II) (29). In the current study, we demonstrated that the T cell responses against the dominant T cell Ag of EV71 (i.e., VP2) were also mainly CD4+ T cell dependent, although determining the roles that these CD4+ T cells played in immunity against EV71 requires further investigation. The incomparable lower CD8+ T cells detected in the current study might also be related with the less efficient expansion of CD8+ T cells against the overlapping peptides than CD4+ T cells (33). Furthermore, functional profiling with IL-2 and IFN-γ revealed that the responding EV71-responsive T cell responses were dominated by single IFN-γ–secreting cells, which may be correlated with the acute viral infection and high viral load with quick disease progression characteristics of primary EV71 infection and recurrent epidemics (57). Nonetheless, a complete understanding of the protection efficiency of VP2–specific T cells during natural infection and vaccination requires additional research in the future.

A major concern in the current study might be that whether the T cell responses detected were elicited from nature EV71 exposure, or cross-primed by other enterovirus infection or polioviruses vaccination, especially considering that a proportion of study subjects showed neutralizing titers to both EV71 and CV-A16, and coinfection with EV71 and CV-A16 has been identified in previous studies (39, 58). Serial analyses were performed to elucidate this question based on the following findings. First, the neutralizing...
Abs detected in the study subjects showed EV71 superior responses to CV-A16, which ensured that the investigation of EV71-specific T cell responses among these subjects was reasonable and reliable. Second, conservancy analysis of the immunogenic peptides identified in the current study demonstrated that peptides with moderate variations of two to four amino acid differences compared with CV-A16 constituted the largest proportion of the immunogenic peptides against EV71 and dominated the responses among the study population. In addition, determination of the cross-reactivity from a representative peptide in CV-A16 suggested that cross-reactivity might have broadly existed among the population, although the variant peptides from CV-A16 were less efficient in priming T cell responses than that from EV71. The partially cross-recognized variant peptides from CV-A16 might provide partial protection against CV-A16 and contribute to a less prevalence of CV-A16 among population (31). Third, although cross-reactivity of variant peptides with three amino acids from polioviruses could be detected from previous study (29), we found that peptides with moderate variations of three or four amino acids occupies ~20% of all the immunogenic peptides from EV71. Furthermore, highly varied peptides with an average of 8.9 aa differences take over >80% of the immunogenic peptides and dominate the responses against EV71. Thus, the influences of previous poliovirus vaccination to EV71-responsive cellular immunity would be limited. Besides, millions of EV71 infections and complications in children despite the programmed nationwide poliovirus vaccination also indicated that poliovirus vaccination contributed little to anti-EV71 immunity (31). However, further exploration is still needed to determine the contribution of cross-reactive T cell responses elicited from natural infection of EV71 or other enteroviruses to the control of disease.

In conclusion, this is, to our knowledge, the first comprehensive study of EV71-responsive cellular immunity comparing the profile of all of the structural Ags. We demonstrated that EV71-responsive T cells broadly exist in healthy adults, and our findings concerning the predominant immunogenicity of the VP2 Ag provide significant implications for the understanding of EV71 cellular immunity. Furthermore, the broadly existed less efficient cross-reactivity against CV-A16 and limited influences from poliovirus vaccination to EV71-responsive cellular immunity would contribute to the understanding of immune protection mechanisms against different enteroviruses.

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Disclosures
The authors have no financial conflicts of interest.
References


