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The Immunodominant CD8 T Cell Response to the Human Cytomegalovirus Tegument Phosphoprotein pp65<sub>495–503</sub> Epitope Critically Depends on CD4 T Cell Help in Vaccinated HLA-A*0201 Transgenic Mice

Michael Reiser,* 1 Andreas Wieland,* 1,2 Bodo Plachter, † Thomas Mertens, ‡ Jochen Greiner, § and Reinhold Schirmbeck*

Immunodominance hierarchies operating in immune responses to viral Ags limit the diversity of the elicited CD8 T cell responses. We evaluated in I-A<sup>B</sup>/A2-IIHD-II and HLA-DR<sup>1</sup>/A2-DR1 mice the HLA-A*0201–restricted, multispecific CD8 T cell responses to the human CMV tegument phosphoprotein pp65 (pp65) Ag. Vaccination of mice with pp65-encoding DNA elicited high IFN-γ+ CD8 T cell frequencies to the pp65<sub>495–503</sub>/e6 epitope and low responses to the pp65<sub>320–328</sub>/e3 and pp65<sub>522–530</sub>/e8 epitopes. Abrogation of the e6-specific immunity efficiently enhanced e3- and e8-specific T cell responses by a pp65<sub>501–503</sub> DNA vaccine. The immunodominant e6-specific (but not the e3- and e8-specific) CD8 T cell response critically depends on CD4 T cell help. Injection of monospecific DNA- or peptide-based vaccines encoding the e3 or e8 (but not the e6) epitope into mice elicited CD8 T cells. Codelivering the antigenic peptides with different heterologous CD4 T cell helper epitopes enhanced e6-specific (but not e3- or e8-specific) CD8 T cell responses. Similarly, homologous CD4 T cell help, located within an overlapping (nested) pp65<sub>487–503</sub> domain, facilitated induction of e6-specific CD8 T cell responses by peptide-based vaccination. The position of the e6 epitope within this nested domain is not critical to induce the immunodominant, e6-specific CD8 T cell response to the pp65 Ag. Distant CD4 T cell epitope(s) can thus provide efficient help for establishing pp65-e6 immunodominance in vaccinated mice. These results have practical implications for the design of new T cell-stimulating vaccines.

Human CMV (HCMV) seroprevalence worldwide ranges between 40 and 90%, depending on the socioeconomic status of the infected population. After primary infection, HCMV establishes latency that can be interrupted by transient reactivations (1). HCMV infections are tightly controlled by the immune system in immunocompetent individuals but may induce life-threatening diseases in immunocompromised patients (e.g., patients with AIDS, organ or hematopoietic stem cell transplant recipients) (2–4). HCMV is frequently transmitted to a developing fetus after primary infection during pregnancy and represents the most significant viral cause of birth defects in developed countries (5). Prophylactic and/or therapeutic vaccines against HCMV thus have a high priority but are not yet available (6–8).
TLR-triggered) help from cells of the innate immune system (38). Interestingly, some MHC class I-restricted epitopes are nested within CD4 T cell epitopes, suggesting that induction of these CD8 T cell responses is facilitated by a closely associated CD4 T cell helper function (33, 34, 39–41). Little is known how CD4 T cells help particular dominant or subdominant CD8 T cell responses, and whether the (distant versus nested) position of the CD8 T cell epitope relative to a CD4 T cell epitope within a large Ag is critical for modulating the respective CD8 T cell specificities.

We used DNA- and peptide-based vaccines to study the CD4 T cell dependence of multispecific and monospecific CD8 T cell responses to the pp65 Ag of HCMV in HLA-A*0201–transgenic (tg) mice [i.e., I-A^b/–A2-HHD-II mice (42) or I-A^b/–/HLA-DR1/ A2-DR1 mice (43)]. The immunodominant, HLA-A*0201–restricted pp65_285–303 epitope is positioned in the pp65 Ag within an overlapping (nested) CD4 T cell helper domain (44–46). We generated DNA-based vaccines encoding wild-type pp65 or mutant pp65 Ags, in which the pp65_285–303 epitope is deleted and/or cloned into a different position within the pp65 protein, and compared CD8 T cell frequencies with eight HLA-A*0201–restricted pp65 epitopes (e1–e8). We further used monospecific, peptide-based vaccines (47), encoding a 1:1 ratio of CD4 and CD8 T cell epitopes as a platform to characterize the specific effects of CD4 help on a distinct CD8 T cell specificity. We used a sensitive assay (i.e., specific IFN-γ induction in a 4-h ex vivo restimulation of CD8 T cells with antigenic peptides) to quantitatively determine the magnitude of specific CD8 T cell responses to pp65.

Materials and Methods

Mice

HLA-A*0201 tg A2-HHD-II (42) and A2-DR1 mice (43) were bred and kept under standard pathogen-free conditions in the animal colonies of Ulm and Mainz universities. Male and female mice were used at 7–10 wk of age. All animal experiments were performed according to the guidelines of the local Animal Use and Care Committee and the National Animal Welfare Law.

MHC I stabilization assays

Cell surface stabilization of HLA-A*0201 molecules was analyzed in the TAP-deficient T2 cell line. In brief, 10^5 T2 cells were incubated overnight with 100 μg/ml peptide in serum-free medium (UltraCulture medium; catalog no. BE 12-725F; Lonza, Braine-l’Alleud Belgium). Cells were washed and stained with FITC-labeled mAb BB7.2 (anti-HLA*A0201, catalog no. BE 12-725F; Lonza) at 37˚C with 5 μg/ml of the indicated peptides. Brefeldin A (catalog no. 15870; Sigma, Taufkirchen, Germany) was added to a final concentration of 5 μg/ml, and the cultures were incubated for 4 h. Cells were harvested, washed, and surface stained with PE-conjugated anti-CD8 mAb (catalog no. 553033; BD Biosciences). Surface-stained cells were fixed with 2% paraformaldehyde in PBS. Fixed cells were resuspended in permeabilization buffer (PBS, 0.5% BSA, 0.5% saponin, 0.05% sodium azide) and incubated with FITC-conjugated anti-IFN-γ mAb (catalog no. 554411; BD Biosciences) for 30 min at room temperature and washed twice in permeabilization buffer. Stained cells were resuspended in PBS/0.3% w/v BSA supplemented with 0.1% w/v sodium azide, and frequencies of IFN-γ^+ CD8^+ T cells were determined by FCN analyses.

Statistics

Data were analyzed using PRISM software (GraphPad, San Diego, CA). The statistical significance of differences in mean CD8 T cell frequencies between groups was determined by using the unpaired Student t test. A p value <0.05 was considered significant.

Results

HCMV/pp65-specific CD8 T cell responses in vaccinated HLA-A*0201 tg mice

We compared binding of eight published epitopes (e1–e8) of the pp65 Ag of HCMV to their restricting MHC class I molecule HLA-A*0201 (Fig. 1A, 1D) (12–16). TAP-deficient T2 cells were pulsed with individual peptides and cell surface stabilization of the loaded HLA-A*0201 molecules was determined. The C18–27 peptide of the hepatitis B core Ag (HBcAg; HBV/C18) that binds HLA-A*0201 with high affinity was included as a positive control (Fig. 1A). The HBV/C18 peptide induced a 4-fold increase in surface HLA-A*0201 expression (Fig. 1B). Similarly, the pp65-derived peptides e1, e3, e5, e6, and e8 efficiently stabilized HLA-A*0201 molecules on the cell surface of T2 cells (Fig. 1B). No or inefficient HLA-A*0201-stabilization was detectable with the peptides e2, e4, and e7 (Fig. 1B). To determine the off rate of HLA-A*0201–bound peptides, we pulsed T2 cells with saturating amounts of the respective peptides at 27˚C and subsequently cultured cells at 37˚C for 1–4 h to force the decay of unstable (empty) HLA-A*0201 cell surface molecules (48). The pp65-derived peptides e3, e6, e8, and the HBV/C18 control peptide showed a comparable slow off rate (Fig. 1C). In contrast, surface expression of HLA-A*0201 molecules binding the e1 and e5 peptides was unstable (Fig. 1C), indicating a faster off rate of these peptides. Thus, the selected pp65 peptides varied in their capacity to bind/stabilize HLA-A*0201 molecules.

DNA-based immunization of HLA-A*0201 tg A2-HHD-II mice (42) was used to evaluate the ability of the eight pp65 epitopes to prime HCMV-specific CD8 T cell responses. The pp65-coding sequence (strain AD169) was inserted into the pCI expression vector.
vector with a streptavidin-binding tag (st) fused NH₂ terminally to the pp65 sequence. Expression of the st-pp65 fusion protein by pCI/st-pp65 DNA was confirmed in transiently transfected HEK cells (Fig. 2A, lanes 2, 3). A2-HHD-II mice were immunized with pCI, pCI/st-pp65, or control pCI DNA. Splenocytes were harvested at day 12 postpriming (i.e., a time point when clonal expansion of DNA-primed CD8 T cells reaches maximal levels) (49, 50) and restimulated ex vivo for 4 h with the respective pp65 peptides. The numbers of CD8 T cells showing specifically inducible IFN-γ expression were determined by FCM (Fig. 2B). The pCI/st-pp65 (but not pCI) vaccination elicited high e6-specific CD8 T cell frequencies and low responses to the e3 and e8 epitopes (Fig. 2B). In this experimental setting, all other peptides tested (e1, e2, e4, e5, and e7) did not stimulate CD8 T cells (Fig. 2B). Only a fraction of the HLA-A*0201–restricted epitope repertoire of pp65 could thus be specifically induced by the pCI/st-pp65 vaccine in mice. Notably, detection of e3, e6, and e8 epitopes in this experimental approach correlated with the high HLA-A*0201 binding/stabilization capacity of these epitopes (Figs. 1B, 1C, 2B).

**Immunodominance hierarchies operate in multispecific CD8 T cell responses to the HCMV/pp65 Ag in HLA-A*0201 tg mice**

The repertoire diversity of multispecific CD8 T cell responses to different epitopes of an Ag restricted by the same MHC class I molecule can be strikingly reduced by immunodominant responses (18, 19). To test whether the e6-specific immunodominant response to pp65 limits HLA-A2*0201–restricted responsiveness to other epitopes of pp65, we constructed a pp65-encoding vaccine in which this epitope was partially deleted. The nine-residue e6 epitope (pp65 495–503; NLVPMVATV) contains an optimal HLA-A2*0201–binding motif, that is, an L at the P2 anchor position and a V at the COOH-terminal P9 anchor position (51).

**FIGURE 1.** HLA-A*0201–restricted epitopes of the HCMV/pp65 Ag. A, For our analyses, we selected eight HLA-A*0201–restricted HCMV/pp65 epitopes (e1–e8). Their positions within the 561-residue pp65 Ag and their sequences are shown. B and C, In vitro HLA-A*0201 I-binding/stabilization properties of the respective pp65 peptides. B, T2 cells were pulsed overnight with 100 μg/ml of the individual peptides, and cell surface expression of HLA-A*0201 molecules was determined by FCM analyses. The stabilization of cell surface HLA-A*0201 molecules by individual peptides is plotted as percentage of mean fluorescence intensity (MFI) compared with nonpulsed T2 cells (set as 100%). C, T2 cells were pulsed overnight with 50 μg/ml of the indicated peptides at 27°C, washed and shifted to 37°C for 1–4 h, followed by cell surface staining of HLA-A*0201 molecules. The decay of cell surface-associated HLA-A*0201 molecules is plotted as MFI at the indicated time points after the temperature shift to 37°C, compared with the MFI before the temperature shift (100%).

**FIGURE 2.** Expression and immunogenicity of HCMV/pp65. A, HEK293 cells were transiently transfected with the noncoding plasmids pCI (lane 1), pCI/st-pp65 (lanes 2, 3), or pCI/st-pp65D_{501–503} (lane 4). Cells were labeled with [35S]methionine, lysed, and the respective Ags were immunopurified with Strep-Tactin Sepharose, followed by release of Ag complexes with SDS. Samples were analyzed by SDS-PAGE and fluorography of the gels. The position of the pp65 Ags is indicated. B, HHD-II mice (four mice per group) were immunized with pCI, pCI/st-pp65, or pCI/st-pp65D_{501–503} DNA. Twelve days after injection, epitope-specific CD8 T cell responses were determined by 4-h ex vivo stimulation of splenic CD8 T cells with the eight pp65-epitopes, followed by determination of IFN-γ⁺ CD8 T cell frequencies. Mean percentages of IFN-γ⁺ CD8 T cells (±SD) of a representative experiment are shown.

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**TABLE 1.** HCMV/pp65 epitopes (HLA-A*0201)

<table>
<thead>
<tr>
<th>Epitope #</th>
<th>Position</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>e1</td>
<td>14-22</td>
<td>VLGPITGSHV</td>
</tr>
<tr>
<td>e2</td>
<td>218-236</td>
<td>VIGDQYYKV</td>
</tr>
<tr>
<td>e3</td>
<td>320-328</td>
<td>LMINGQQIFL</td>
</tr>
<tr>
<td>e4</td>
<td>482-491</td>
<td>FFTWPWQAGI</td>
</tr>
<tr>
<td>e5</td>
<td>491-500</td>
<td>ILARNLVPMV</td>
</tr>
<tr>
<td>e6</td>
<td>495-503</td>
<td>NLVPMVATV</td>
</tr>
<tr>
<td>e7</td>
<td>509-517</td>
<td>KYQJFFWD</td>
</tr>
<tr>
<td>e8</td>
<td>522-530</td>
<td>RIFAKELEGV</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>HBV/C18</td>
</tr>
<tr>
<td></td>
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delected three amino acids at the positions pp65<sub>501–503</sub> (NLVPMVATV) to generate the pp65 mutant st-pp65<sub>501–503</sub>. Transient transfection of HEK cells confirmed expression of the st-pp65<sub>501–503</sub> Ag (Fig. 2A, lane 4). A2-HHD-II mice were immunized with this pCI/st-pp65<sub>501–503</sub> vaccine, and their specific responses to the pp65 epitopes were read out 12 d postpriming (Fig. 2B). Specific ex vivo restimulation of primed splenocytes with the e6 peptide did not expand IFN-γ<sup>+</sup> CD8 T cells (Fig. 2B), confirming that the pp65<sub>501–503</sub> mutation in the pp65 Ag destroyed the antigenicity of the e6 epitope. Deletion of the e6 epitope from the pp65 vaccine significantly improved e3- and e8-specific CD8<sup>+</sup> T cell responses in pCI/st-pp65<sub>501–503</sub>-immunized mice (Fig. 2B). Four- to 10-fold higher numbers of e3- and e8-specific CD8<sup>+</sup> T cells were induced by the pCI/st-pp65<sub>501–503</sub> as compared with the pCI/st-pp65 vaccine (Fig. 2B). These findings suggested that the dominant, HLA-A*0201–restricted CD8<sup>+</sup> T cell response to e6 suppressed CD8<sup>+</sup> T cell responses to the e3 and e8 epitopes. CD8<sup>+</sup> T cell responses against the e1, e2, e4, e5, and e7 epitopes were not detected in pCI/st-pp65<sub>501–503</sub> vaccinated mice (Fig. 2B).

**Induction of monospecific CD8 T cell responses to individual, HLA-A*0201–restricted pp65 epitopes**

To characterize CD8<sup>+</sup> T cell responses to individual e3, e6, and e8 epitopes in mice, we generated epitope-monospecific, DNA-, or peptide-based vaccines. We constructed DNA vaccines encoding the e3, e6, or e8 epitope by inserting their coding sequences COOH terminally to an endoplasmic reticulum (ER)-targeting sequence (METDTLLLWVLWWPGSTGD) of the murine Igκ-chain. The ER-targeted epitopes are released by signal peptidases in the ER without further processing (52, 53). We previously showed that ER-targeted epitope vaccines efficiently elicit monospecific CD8<sup>+</sup> T cell responses (54). We constructed three vectors: pCI/L-e3, pCI/L-e6, and pCI/L-e8. Whereas the injection of the pCI/L-e3 and pCI/L-e8 DNA vaccines into A2-HHD-II mice efficiently elicited e3- and e8-specific CD8<sup>+</sup> T cell responses (Fig. 3A, groups 2, 4), the pCI/L-e6 vaccine did not prime (or very inefficiently primed) e6-specific CD8<sup>+</sup> T cells (Fig. 3A, group 3).

We further used a recently developed peptide immunization protocol to induce monospecific CD8<sup>+</sup> T cell responses against these pp65 epitopes (47). We complexed immunostimulatory ODNs to short, synthetic peptides in which the cationic HIV-tat<sub>50–57</sub> RKKRRQRRRR domain (tat) was fused NH<sub>2</sub> terminally to the antigenic e6, e3, and e8 epitopes (47). This generated the cationic tat/e3 (tat-LMNGQQIQFL), tat/e6 (tat-NLVPMVATV), and tat/e8 (tat-RIFAELGEV) peptides. Peptide vaccines were injected into A2-HHD-II mice (Fig. 3B). Injection of tat/e3 and tat/e8 into A2-HHD-II mice induced e3- and e8-specific CD8<sup>+</sup> T cell responses (Fig. 3B, groups 2, 4), whereas the tat/e6 vaccine did not elicit e6-specific CD8<sup>+</sup> T cells (Fig. 3B, group 3). Notably, neither the noncomplexed cationic peptides (without ODN) nor the antigenic peptides (without the cationic tat sequence) co-delivered with ODN elicited CD8<sup>+</sup> T cell responses in A2-HHD-II mice (data not shown). Minimal epitope vaccines containing the pp65-derived e3 or e8 epitope, but not those containing the immunodominant e6 epitope, can thus efficiently induce monospecific CD8<sup>+</sup> T cell responses if complex formation between the cationic/antigenic peptide and ODN is used. Thus, nonspecific ODN-mediated help from cells of the innate immune system (38) facilitates priming of e3- and e8-specific (but not e6-specific) CD8<sup>+</sup> T cell responses.

**Priming of e6-specific CD8 T cell responses critically depends on CD4 T cell help**

It has been shown that CD4 T cell help is important for the induction and maintenance of CD8<sup>+</sup> T cells (25). We asked whether pp65-specific, homologous CD4<sup>+</sup> T cell help modulates induction of the respective CD8<sup>+</sup> T cell specificities. We eliminated CD4<sup>+</sup> T cells by treatment with the depleting Ab YTS-191 before and after pCI/st-pp65-specific DNA vaccination of A2-HHD-II mice. In comparison with control Ab-treated mice, CD4<sup>+</sup> depletion resulted in a significant decrease of e6-specific IFN-γ<sup>+</sup> CD8<sup>+</sup> T cell frequencies (Fig. 3C). In contrast, CD8<sup>+</sup> T cell numbers specific for the subdominant e3 and e8 epitopes even tend to slightly increase in CD4-depleted mice but did not reach the levels primed by the e6-deficient pCI/st-pp65<sub>501–503</sub> vaccine (Figs. 2B, 3C). These findings showed that the manifestation of the e6-specific (but not the e3- or e8-specific) CD8<sup>+</sup> T cell response depends on CD4<sup>+</sup> T cell help. The reduced number of e6-specific CD8<sup>+</sup> T cells can suppress subdominant e3- and e8-specific CD8<sup>+</sup> T cell responses in CD4<sup>+</sup> T cell-depleted mice (Fig. 3C). The number of immunodominant CD8<sup>+</sup> T cells is apparently not important to suppress subdominant immune responses, confirming our previous reports in an HBV model (55, 56).

Monospecific, peptide-based vaccines delivering the e6 epitope of pp65 and MHC class II-binding, heterologous pan DR epitope- or tetanus toxin-derived CD4<sup>+</sup> T cell helper epitopes have been shown to efficiently induce CD8<sup>+</sup> T cell responses in HLA-A*0201 tg mice (32, 35). The sequence N terminal to and comprising the
e6/pp65_{495-503} epitope encodes a class II-binding (HLA-DR4/11; pp65_{485-503}; Fig. 1D) domain (44–46). CD4 T cell epitopes could be promiscuous in their HLA-DR and I-A\(^b\) binding (57–60). We thus evaluated whether the nested CD4/CD8 T cell domain supports priming of e6-specific CD8 T cell responses in A2-HHD-II mice (expressing the murine I-A\(^b\) class II molecule). We \(\text{NH}_2\) terminally extended the tat/e6 peptide by four (tat/e6+n4) or eight (tat/e6+n8) pp65-specific amino acids (Fig. 4A) and immunized A2-HHD-II mice with the respective cationic peptide/ODN complexes. Injection of the tat/e6+n8 vaccine (but not the tat/e6 or tat/e6+n4 vaccines) efficiently elicited high frequencies of e6-specific IFN-\(\gamma\) CD8 T cells (Fig. 4B, groups 1–3), indicating that the N-terminal flanking sequence of the e6 epitope plays an important role in priming e6-specific CD8 T cells. Similarly, N-terminal fusion of a well-defined, I-A\(^b\)-binding HBV Core_{128-142} (C128) sequence to the e6+n4 peptide (Fig. 4A, tat/e6+n4/C128) efficiently elicited e6-specific CD8 T cell responses in A2-HHD-II mice (Fig. 4B, group 4).

It is not critical to deliver the antigenic e6 epitope and the helper sequence as a cationic fusion peptide to induce e6-specific CD8 T cells. Injection of a mixture of two cationic tat/e6+n4 and tat/C128 peptides (complexed with ODN) into A2-HHD-II mice efficiently induced e6-specific CD8 T cells (Fig. 4C, group 2), confirming previous reports (32, 35). In contrast, e6-specific CD8 T cell responses were not induced when the respective tat/e6+n4 and tat/C128 vaccines were injected into different sites (i.e., into the right and left tibialis anterior muscles) of A2-HHD-II mice (Fig. 4C, group 3). Hence the local copriming of C128-specific CD4 T cell help allowed priming of e6-specific CD8 T cells. There is thus no absolute requirement that help must come via recognition of an overlapping or nested CD4 T cell epitope. To evaluate whether the I-A\(^b\)-restricted, C128-specific CD4 T cell help can improve subdominant, e8-specific CD8 T cell responses, we fused the I-A\(^b\)-binding C128 sequence \(\text{NH}_2\) terminally to the e8 epitope (Fig. 4A). This tat/e8/C128 vaccine efficiently induced e8-specific CD8 T cells in A2-HHD-II mice but, in comparison with the tat/e8 vaccine, C128-specific help did not enhance the e8-specific CD8 T cell response (Fig. 4D, groups 2, 3). Similarly, the tat/e3 and the tat/e3/C128 vaccines elicited comparable e3-specific CD8 T cell frequencies (data not shown). I-A\(^\alpha\)–restricted heterologous CD4 T cell help is thus important to induce e6-specific (but not e3- or e8-specific) CD8 T cell responses in A2-HHD-II mice.

**An immunodominant, e6-specific immunity is induced in HLA-A*0201/HLA-DR1\(^\text{tg}\) (A2-DR1) mice**

We next used HLA-A*0201-tg A2-DR1 mice (expressing the human HLA-DR1 but not the murine I-A\(^\alpha\) class II molecules) (43) to evaluate whether the pp65-specific CD8 T cell immunity varies in mice with a different CD4 T cell helper repertoire. Immunization of A2-DR1 mice with pCI/st-pp65 DNA elicited a prominent e6-specific CD8 T cell response and low responses to the e3 and e8 epitopes (Fig. 5A). Inactivation of the e6 epitope in the pCI/pp65_{495-503} vaccine resulted in a significantly increased e3- and e8-specific CD8 T cell immunity (Fig. 5A). CD8 T cell responses against the e1, e2, e4, e5, and e7 epitopes were not detectable in vaccinated A2-DR1 mice (data not shown). A comparable CD8 T cell response against pp65 and mutant pp65_{495-503} could thus be specifically induced in A2-HHD-II and A2-DR1 mice by DNA-based vaccines (Figs. 2B, 5A).

Similarly, the peptide-based tat/e6+n8 vaccine (but not the tat/e6 and tat/e6+n4 vaccines) efficiently elicited high frequencies of e6-specific IFN-\(\gamma\) CD8 T cells in A2-DR1 mice (Fig. 5B, groups 1–3), suggesting that a homologous pp65-intrinsic help facilitated priming of e6-monospecific CD8 T cell responses in these mice. The I-A\(^\alpha\)-specific help codelivered by the tat/e6+n4/C128 vaccine did not elicit e6-specific CD8 T cell responses in A2-DR1 mice (Fig. 5B, group 4). C128-specific, I-A\(^\alpha\)-restricted CD4 T cell help thus operated in I-A\(^\alpha\)-expressing A2-HHD-II but not in HLA-DR1–expressing A2-DR1 mice (Figs. 4B, 5B, group 4). To confirm that a CD4 T cell helper function of e6-encoding antigenic peptides is critical for inducing e6-specific immunity, we repeated the experiment using tat/e6+n4/C128 and tat/e6+C128 vaccines in A2-DR1 mice (Fig. 5B, group 5). This tat/e6+C128 vaccine efficiently induced e6-specific CD8 T cell frequencies, as did tat/e6+n4/C128, whereas tat/e6+n4/C128 vaccine did not elicit e6-specific CD8 T cell frequencies (Fig. 5B, group 6). This suggests that the tat/e6+C128 vaccine included an e6-specific CD8 T cell helper function (Fig. 5B, group 5).

**FIGURE 4.** CD4 T cell help facilitates induction of e6-monospecific CD8 T cell responses in HLA-A*0201 tg mice. Sequences of cationic peptides that are complexed with ODN-1826 and used in the vaccination studies (A). A2-HHD-II mice (three to four mice per group) were immunized (B) with tat/e6 (group 1), tat/e6+n4 (group 2), tat/e6+n8 (group 3), tat/e6+n4/C128 (group 4); or (C) were either not immunized (group 1), immunized with a mixture of tat/C128 and tat/e6+n4 (group 2), or immunized with tat/C128 (into the left muscle) and cp/pp65–e6+n4 (into the right muscle; group 3); or immunized (D) with tat/e6 (group 1) or tat/e8/C128 (group 2) vaccines. Mice were boosted after 3 wk with the respective vaccines. Twelve days after the last injection, CD8 T cell responses were determined by 4-h ex vivo stimulation of splenic CD8 T cells with the e3, e6, and e8 peptides, followed by determination of IFN-\(\gamma\) CD8 T cell frequencies. Mean percentages of IFN-\(\gamma\) CD8 T cells (± SD) of a representative experiment are shown.
Twelve days after injection, IFN-γ CD8 T cell frequencies were determined by 4-h ex vivo stimulation of spleen cells with the e3, e6, and e8 peptides. Mean percentages of IFN-γ CD8 T cells (±SD) of a representative experiment are shown. The statistical significance of differences between the e3- and e8-peptide–stimulated groups was determined by the unpaired Student t test (*p < 0.05). B: A2-DR1 mice (three mice per group) were immunized with tat/e6 (group 1), tat/e6+n4 (group 2), tat/e6+n8 (group 3), tat/e6+n4/C128 (group 4), or tat/e8/TT830 vaccines (group 5). Mice were boosted after 3 wk with the respective vaccines. Twelve days after the last injection, CD8 T cell responses were determined by 4-h ex vivo stimulation of splenic CD8 T cells with the e6 or a HLA-A*0201–binding HBV/C18 epitope (control), followed by determination of IFN-γ CD8 T cell frequencies. Mean percentages of IFN-γ CD8 T cells (±SD) of a representative experiment are shown.

Priming of immunodominant e6-specific CD8 T cells does not depend on the position of the e6 sequence within the pp65 Ag

To evaluate whether the nested CD4 T cell helper function is important for the induction of the immunodominant e6-specific CD8 T cell response to pp65, we constructed pp65 mutant, which encodes the e6 epitope in a different position of the pp65 Ag. We used the pCI/st-pp65Δ501–503 construct (in which the e6 epitope is inactivated; see Fig. 2), deleted the e1/pp6514–22 epitope (which did not elicit a specific CD8 T cell immunity in A2-tg mice; see Figs. 1A, 2), and cloned the minimal e6 epitope–encoding sequence into this position (Fig. 6A). This generated the pCI/st-pp65Δ501–503Δe1/e6 vector (Fig. 6A). Transient transfection of HEK cells confirmed expression of the st-pp65Δ501–503Δe1/e6 Ag (Fig. 6B, lane 4). A2-DR1 mice were immunized with the pCI/st-pp65Δ501–503Δe1/e6 DNA, and specific CD8 T cell immunity was read out 12 d postpriming (Fig. 6C). The pCI/st-pp65Δ501–503Δe1/e6 vaccine efficiently elicited e6-specific (but low e3- and e8-specific) CD8 T cell frequencies (Fig. 6C). The pp65-specific CD8 T cell response was comparable in pCI/st-pp65 and pCI/st-pp65Δ501–503Δe1/e6 immunized A2-DR1 mice (Fig. 6C), and e3- and e8-specific CD8 T cell frequencies were significantly lower compared with those primed by the e6-deficient pCI/st-pp65Δ501–503 vaccine (Figs. 5A, 6C). Induction of an e6-specific CD8 T cell immunity thus correlated with the suppression of e3- and e8-specific immune responses, confirming that e6-specific immunodominance mechanisms operate in A2-tg mice. The position of the e6 epitope within the mutant st-pp65Δ501–503Δe1/e6 Ag and its flanking sequences are thus not critical to induce e6-specific CD8 T cells.

Discussion

Vaccines are designed to prime multispecific CD8 T cell responses that can mediate protective immunity against different intracellular pathogens (61). However, interference between responses to individual epitopes presented by the same or different MHC class I molecules is the well-established phenomenon of immunodominance that limits the repertoire diversity of CD8 T cell responses primed by such vaccines (18, 19). We have shown that immunodominance hierarchies operate in CD8 T cell responses to different epitopes of the same Ag, between different Ags of the same virus, or between unrelated Ags (50, 55, 56, 62, 63). A single immunodominant epitope can efficiently suppress many other CD8 T cell responses to epitopes of the same or different Ags. For example, an immunodominant L1-restricted epitope from the hepatitis B surface Ag (HBsAg) suppressed multispecific CD8 T cell responses to Kd, Dd, and Kβ-restricted epitopes of this viral Ag, and inactivation of this immunodominant epitope by a single amino acid exchange within the HBsAg efficiently restored priming of multiple subdominant CD8 T cell responses (56). We showed in this study that the major CD8 T cell response to the HCMV/pp65 Ag in vaccinated HLA-A*0201 tig mice is directed against the e6/pp65Δ501–503 epitope. Inactivation of the e6
epitope from the pp65 vaccine (st-pp65Δ501–503) significantly enhanced CD8 T cell responses against two other (e3 and e8) epitopes of pp65, indicating that the e6-specific immunity suppresses these CD8 T cell specificities.

Different factors have been proposed to contribute to immunodominance, such as competition between epitopes to bind to the same MHC class I molecules (64, 65) or competition between different MHC class I alleles for cell surface expression (66). The HLA-A*0201 tg mice used in this study express only the human HLA-A*0201 MHC class I molecule (42, 43). Simple differences in HLA-A*0201 binding by the dominant e6 and the subdominant e3 and e8 peptides does not explain the observed immunodominance in HCMV/pp65-immune mice because all three peptides have a similar HLA-A*0201–binding/stabilization capacity (see Fig. 1B, 1C). Using Ab-mediated CD4 T cell depletion of pp65-immune mice, we showed that the immunogenicity of the immunodominant e6 (but not the e3 and e8) epitope depends on CD4 T cell help. CD4 T cells play a major role in establishing and sustaining CD8 T cell responses. We did not detect differences in the kinetics of e6-, e3-, and e8-specific CD8 T cell responses (data not shown). The DNA-primed CD8 T cell responses peaked at 12–14 d postvaccination and declined to low levels in the following 20–30 d as previously reported for other Ags (49, 50). We therefore assume that CD4 T cell help facilitates priming of e6-specific CD8 T cells in pp65-immune mice, which limit e3 and e8 CD8 T cell responses by a yet unknown mechanism.

It is yet unknown why many (but not all) CD8 T cell responses require CD4 T cell help. We showed that different sources of help facilitate priming of immunodominant (e6) and subdominant (e3 and e8) CD8 T cell responses. Monospecific DNA- or peptide/ODN-based vaccines efficiently elicited e3- and e8-specific (but not e6-specific) CD8 T cell responses in HLA-A*0201 tg mice. CpG-containing ODNs have been shown to provide TLR9-dependent, immunostimulatory signals to the innate immune system (38). Similarly, plasmid DNA induced immunostimulatory immune responses in mice (67). This nonspecific innate help is thus sufficient to induce e3- and e8-specific (but not e6-specific) CD8 T cells. In contrast, priming of e6-specific CD8 T cell responses by peptide-based vaccines critically depends on heterologous CD4 T cell help. An eight-residue, N-terminal extension of e6 epitope from the pp65 vaccine (st-pp65Δ501–503) significantly enhanced CD8 T cell responses against two other (e3 and e8) epitopes of pp65, indicating that the e6-specific immunity suppresses these CD8 T cell specificities.

In preliminary experiments, we have evaluated the interference between CD8 T cell responses may be more complex in the HCMV infection of humans because more Ags are expressed and more antigenic epitopes compete for binding/presentation of different MHC class I and II molecules (73). Moreover, persistent viral infections and reactivations may critically affect the longevity of antiviral CD8 T cell responses (50, 74, 75). In preliminary experiments, we have evaluated the repertoire of pp65-specific CD8 T cell responses in four HCMV-seropositive HLA-A*0201+ volunteers. All individuals showed strong CD8 T cell responses to the pp65-e6 epitope, whereas responses to all seven other pp65 epitopes were not detectable by granzyme B or IFN-γ ELISPOT assays (data not shown). The CD8 T cell response to the e6 epitope was thus immunodominant in both HCMV-infected humans and HCMV/pp65-vaccinated mice.

Little is known about how immunodominant and subdominant determinants are distinguished by the CD8 T cell system. Protective immunity often does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides (30, 55, 62, 76, 77). We previously showed that a subdominant, HBeAg-specific, but not a dominant HBSAg-specific, CD8 T cell response was efficiently elicited in
transgene mice that harbor a replicating HBV genome in the liver (62). HBCAg-specific CD8 T cells accumulated in the liver of vaccinated mice and transiently suppressed HBV replication (62). Subdominant epitopes in vaccines can hence prime specific CD8 T cell immunity in a tolerogenic milieu that delivers specific antiviral effects to HBV-expressing hepatocytes. Interestingly, priming of dominant HBsAg-specific CD8 T cell responses by HBSag-encoding vaccines is CD4 T cell help dependent (62, 78), whereas priming of the subdominant HBBag-specific CD8 T cell responses by a HBBag-encoding DNA is not. Similarly, we showed in this study CD4 T cell-mediated help in the induction of dominant but not subdominant CD8 T cell responses to HCMV pp65 Ag. These results have practical implications for the design and assessment of new T cell-stimulating vaccines.

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Disclosures

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References
