The Context of Epitope Presentation Can Influence Functional Quality of Recalled Influenza A Virus-Specific Memory CD8+ T Cells

E. Bridie Day, Weiguang Zeng, Peter C. Doherty, David C. Jackson, Katherine Kedzierska and Stephen J. Turner

*J Immunol* 2007; 179:2187-2194; doi: 10.4049/jimmunol.179.4.2187
http://www.jimmunol.org/content/179/4/2187

**References**
This article cites 47 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/179/4/2187.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
The Context of Epitope Presentation Can Influence Functional Quality of Recalled Influenza A Virus-Specific Memory CD8+ T Cells

E. Bridie Day, Weiguang Zeng, Peter C. Doherty, David C. Jackson, Katherine Kedzierska, and Stephen J. Turner

Lipopeptide constructs offer a novel strategy for eliciting effective cellular and humoral immunity by directly targeting the vaccine Ag to dendritic cells. Importantly, it is not known how closely immunity generated after lipopeptide vaccination mimics that generated after natural infection. We have used a novel lipopeptide vaccine strategy to analyze both the quantity and quality of CD8+ T cell immunity to an influenza A virus epitope derived from the acidic polymerase protein (PA224) in B6 mice. Vaccination with the PA224 lipopeptide resulted in accelerated viral clearance after subsequent influenza virus infection. The lipopeptide was also effective at recalling secondary D8PA224 responses in the lung. Lipopeptide recalled D8PA224-specific CTL produced lower levels of IFN-γ and TNF-α, but produced similar levels of IL-2 when compared with D8PA224-specific CTL recalled after virus infection. Furthermore, lipopeptide- and virus-recalled CTL demonstrated similar TCR avidity. Interestingly, lipopeptide administration resulted in expansion of D8PA224-specific CTL using a normally subdominant TCRBV gene segment. Overall, these results demonstrate that protective CTL responses elicited by lipopeptide vaccines can be correlated with TCR avidity, IL-2 production, and broad TCR repertoire diversity. Furthermore, factors that impact the quality of immunity are discussed. These factors are important considerations when evaluating the efficacy of novel vaccine strategies that target dendritic cells for eliciting cellular immunity. The Journal of Immunology, 2007, 179: 2187–2194.

E. Bridie Day, Weiguang Zeng, Peter C. Doherty, David C. Jackson, Katherine Kedzierska, and Stephen J. Turner

Efficient resolution from viral infection often relies on the induction of effective CD8+ cytotoxic T cell (CTL) immunity (1). After infection, viral peptides (p) are presented by the MHC class I (MHCI) glycoproteins on the cell surface in an allele-specific manner (2). Upon recognition of pMHCI complexes on activated dendritic cells (DCs) via the clonally expressed TCR (3), naive CTL undergo a program of differentiation resulting in the acquisition of effector functions and rapid expansion (4–6). After a pathogen has been cleared, CTL numbers contract leaving behind a small population of long-lived memory CTL (4, 6). Memory CTL are characteristically different to naive CTL in that they are now present in higher numbers and display more rapid effector function upon recognition of pMHCI (7). Given the important role for CTL in limiting viral replication and spread, the induction of CTL responses by vaccination is thought to be a means of providing effective protection from the consequences of viral infection (1).

Generally, most current vaccine strategies are based on intact pathogens where the pathogen has been rendered nonpathogenic, either by attenuation or inactivation. The rationale for using a vaccine similar to the “natural” form of the pathogen is the expectation that such vaccines will induce immunity similar to that induced by natural infection. Importantly, while vaccine strategies based on inactivated pathogens are effective at inducing protective Ab responses, they are poor at inducing CTL immunity (8). This is likely due to the inappropriate delivery of Ag to the appropriate Ag-presentation pathway, and the absence of a danger signal required to appropriately activate DCs for priming of CTL responses. Recently, recognition of the importance of DCs in priming effective CTL responses has resulted in vaccine strategies that use either transfer of Ag-loaded DCs (9), or synthetic constructs that target the Ag to DCs in vivo (10).

Synthetic lipopeptide vaccines have demonstrated potential as a novel vaccine strategy for eliciting cellular immunity (11, 12). Their construction consists of minimal peptide determinants (both CD4 and CD8 T cell epitopes) conjugated to a lipid moiety S-[2,3-bis[palmito]loxy]propyl)cysteine (Pam2Cys) derived from Mycoplasma fermentans (13). The Pam2Cys component of the vaccine formulation is a potent activator of DCs via the TLR2 (14, 15). In mouse models, lipopeptide vaccination has provided robust cellular immunity against viral, bacterial, and tumor challenge (8, 12, 16). However, it is unclear how the quality of CTL immunity induced after lipopeptide administration mimics that observed after virus infection. To determine this, a comparison of various immune parameters is warranted as it could reveal novel immune correlates that may be useful in evaluating the efficacy of vaccine strategies.
Respiratory infection of C57BL/6 (H2b) mice with influenza A virus causes an acute, localized pneumonia (17) and results in a CTL response that is directed against at least six viral peptides (18). The most prominent, and most well-characterized, responses are directed against peptides derived from the viral nucleoprotein (NP366–374, H2Db binding; Ref. 19) and the acid polymerase (PA224–236, H2Db binding; Ref. 20). Comparison of these influenza-specific CTLs has demonstrated several quantitative and qualitative differences. For example, during primary infection, the magnitude of the CD8+ T cell response to each of these determinants is roughly equivalent (21–23). However, the DNP366-specific set dominates the response after secondary challenge (20, 23). Functional analysis shows that relatively more CD8+ DPA224+ compared with CD8+ DNP366+ T cells make TNF-α and IL-2 after in vitro peptide stimulation (21, 22, 24). This greater functional capacity also correlates with a higher avidity pMHC interaction (22, 25).

Given the potential of lipopeptide vaccination to elicit enhanced CTL responses and the need for identifying novel immune correlates, we were interested in how lipopeptide vaccination impacted various measures of CTL quality. We investigated the capacity of lipopeptide vaccination to elicit DPA224+-specific responses, and protect from subsequent influenza A virus challenge. We also compared the quantity and functional quality of the DPA224+-specific CTL generated after both lipopeptide vaccination and infection to establish how closely this vaccination strategy mimics the immune quality established after infection.

Materials and Methods

Synthesis and purification of lipitated and nonlipitated epitope-based vaccines

Synthetic peptide and lipopeptide constructs were synthesized as previously described (13). The constructs incorporated an immunodominant H2Db-restricted CD8+ T cell determinant from the acid polymerase of influenza A virus (SSLENFRAYV; PA224) and a CD4+ T cell epitope derived from the fusion protein of the morbillivirus, canine distemper virus (KLIPNASLIENCTKAEL) (26). Peptides were assembled linearly on F-moc-Wang resin (Auspep) using conventional solid-phase F-moc chemistry. The nonlipitated construct Th-PA, contained the CD4+ and CD8+ T cell epitopes, separated in sequence by a lysine residue. The lipitated construct, Th-P2C-PA, incorporated the lipid moiety Pam2Cys, derived from the macrophage-activating lipopeptide 2 isolated from the macrophage. This construct is expressed by human macrophages in the presence of CD40 ligand and induces TNF-α production by CD8+ memory T cells (27).

Inoculation/infection of mice

C57BL/6d (B6, H2b) mice were bred at the University of Melbourne (Parkville, Australia). To study various aspects of the immune response to epitope-based vaccines, a variety of inoculation/infection schedules were used, with mice receiving either a 9 or 45 nmol dosage. For analysis of primary responses, naive B6 mice were anesthetized with methoxyflurane and inoculated intranasally (i.n.) with 10³ PFU influenza virus A/HKx31 (H3x3, H3N2). To study recall responses, mice were primed by i.p. injection with 1.5 × 10⁶ PFU AP/PR8/34 (PR8, H1N1) virus at least 6 wk before inoculation with peptide constructs or secondary challenge with 10⁶ PFU HKx31 virus. For viral challenge experiments, mice were either primed with 1.5 × 10⁶ PFU PR8 virus or inoculated i.n. with peptide constructs. Six weeks postpriming, mice were infected i.n. with 10³ PFU of a mutant HKx31 virus (HKx31 NP NSQ), which contained a glutamic acid substitution at position 5 of the immunodominant viral epitope derived from the influenza A nucleoprotein (NP366) (27). This mutation completely abolishes presentation of the NP366 epitope by H2Dd and was used to assay immune protection in the absence of a DNP366-specific CTL response. Ethics approval for animal experiments was obtained from the University of Melbourne Animal Experimental Ethics Committee.

Virus stocks were grown in the allantoic cavity of day 10 embryonated chicken eggs, and titers were determined by plaque assay as PFU on monolayers of Madin Darby canine kidney cells (8).

Tissue sampling and cell preparation

Spleen and lung lymphocyte populations were recovered from mice at the indicated time points and processed as previously described (22). Brieﬂy, single-cell suspensions were prepared from perfused lungs following enzymatic digestion with collagenase A (Roche Applied Science) and passed through 70-μm cell strainers (BD Biosciences). RBC were lysed by treatment with Tris-buffered ammonium chloride. Spleens were disrupted by grinding between frosted slides and enried for CD8+ cells by incubation on tissue culture dishes coated with goat anti-mouse IgG and goat anti-mouse IgM (Jackson ImmunoResearch Laboratories).

IFN-γ ELISPOT assay

Wells of 96-well Millipore Multiscreen-HA filter plates were coated overnight with 10 μg/ml rat anti-mouse IFN-γ capture Ab (RA-6A2; BD Biosciences/BD Pharmingen). Wells were then blocked with complete RPMI 1640 medium (JRH Biosciences) containing 10% v/v heat-inactivated FCS (JRH Biosciences), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 mM nonessential amino acids, 5 mM HEPES, 55 mM 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (In Vitrogen Life Technologies). Spleen and lung suspensions were prepared and added to wells in 2-fold dilutions at 5 × 10⁵ irradiated (2200 rad) naive splenocytes pulsed with or without 2 μg/ml PA224 peptide (Auesp). IFN-γ production was detected by staining with rat anti-mouse biotinylated IFN-γ detecting Ab (XMG1.2; BD Biosciences/BD Pharmingen) at 5 μg/ml in PBS with 0.05% Tween 20 (APS Chemicals) and 1% heat-inactivated FCS, followed by incubation for 1 h with streptavidin-alkaline-phosphatase diluted 1/500 in PBS with 0.05% Tween 20. ELISPOT substrate containing 1 mg of 5-bromo-4-chloro-3-indolyphosphate (Roche Diagnostics) per milliliter of 2-aminoo-2-methyl-1-propynol buffer (Sigma-Aldrich) was added to the wells and spots were developed at 37°C, 5% CO2 for 30 min. Spots were counted on an AID EliSpot Reader System version 3.1.1 (Autoimmunagnostica).

Stimulation and intracellular cytokine staining

Enriched T cell populations (0.5–2 × 10⁶ cells) from the spleen and lung were stimulated for 5 h in 200 μl of complete RPMI 1640 medium, 10 U/ml recombinant human IL-2 (Roche Diagnostics), together with 5 × 10⁵ irradiated (2200 rad) naive splenocytes pulsed with or without 2 μg/ml PA224 peptide (Auesp). IFN-γ production was detected by staining with rat anti-mouse biotinylated IFN-γ detecting Ab (XMG1.2; BD Biosciences/BD Pharmingen) at 5 μg/ml in PBS with 0.05% Tween 20 (APS Chemicals) and 1% heat-inactivated FCS, followed by incubation for 1 h with streptavidin-alkaline-phosphatase diluted 1/500 in PBS with 0.05% Tween 20. ELISPOT substrate containing 1 mg of 5-bromo-4-chloro-3-indolyphosphate (Roche Diagnostics) per milliliter of 2-aminoo-2-methyl-1-propynol buffer (Sigma-Aldrich) was added to the wells and spots were developed at 37°C, 5% CO2 for 30 min. Spots were counted on an AID EliSpot Reader System version 3.1.1 (Autoimmunagnostica).

Tetramer staining and tetramer dissociation analysis

Lymphocytes (0.5–2 × 10⁶ cells) from the spleen and lung were stained with DPA224 tetramer conjugated to either streptavidin-PE or streptavidin-alkaline phosphatase (OligoTech) for 60 min at room temperature. Cells were washed in FACS Buffer before staining with anti-CD8α-PE (55-1-501; BD Biosciences/BD Pharmingen) for 30 min on ice. After washing, cells were permeabilized by paraformaldehyde fixation, using reagents supplied in a Cytofix/Cytoperm kit (BD Biosciences/BD Pharmingen) according to the manufacturer’s instructions. Cells were stained with anti-IFN-γ-FITC (XMG1.2; BD Biosciences/BD Pharmingen), anti-IL-2-PE (JES6-5H4; BD Biosciences/BD Pharmingen), and anti-TNF-α-allophycocyanin (MP6-XT22; BD Biosciences/BD Pharmingen) and subsequently stained with CD8α-FITC and anti-CD4-PE (BD Biosciences/BD Pharmingen) for 30 min on ice. After washing, cells were analyzed by flow cytometry using a FACSCElara flow cytometer (BD Immunocytochemistry Systems) and data were analyzed using CellQuest Pro software (BD Immunocytochemistry Systems).

Downloaded from http://www.jimmunol.org/ by guest on July 25, 2017
lipopeptide. At the acute time point (day 8), enriched T cell populations from the spleen were stained with D\(^{\text{b}}\)PA224 tetramer, anti-CD8e-allophycocyanin Ab and anti-V\(^{\text{b}}\) FITC Ab. Data show mean numbers of D\(^{\text{b}}\)PA224-specific CD8\(^{\text{b}}\) cells ± SD of four mice (except PBS lung day 9, n = 3 mice). The statistical analysis compares samples to PBS groups on each separate panel (*, p < 0.05; **, p < 0.01).

**FIGURE 1.** Quantitation of primary and memory responses induced by lipopeptide vaccine constructs. Naive B6 mice were inoculated with 10\(^{4}\) PFU X31 virus (i.n.), 45 nmol Th-P2-PA (i.n.), 45 nmol Th-PA (i.n.), or 30 \(\mu\)l of PBS (i.n.). On day 9 (A and B) or 31 (C and D), spleens (A and C) and lungs (B and D) were harvested and single-cell suspensions prepared. D\(^{\text{b}}\)PA224-specific CD8\(^{\text{b}}\) cells were identified by staining with D\(^{\text{b}}\)PA224-PE tetramer and anti-CD8e-FITC Ab. Data show mean numbers of D\(^{\text{b}}\)PA224-specific CD8\(^{\text{b}}\) cells ± SD of four mice (except PBS lung day 9, n = 3 mice). The statistical analysis compares samples to PBS groups on each separate panel (*, p < 0.05; **, p < 0.01).

### Results

**Administration of the Th-P2-CA lipopeptide vaccine elicits primary D\(^{\text{b}}\)PA224-specific CTL responses**

Lipopeptide vaccination has been demonstrated to be effective at providing CTL immunity against viral and bacterial challenge (8, 12, 16). To demonstrate that a lipopeptide vaccine construct could elicit D\(^{\text{b}}\)PA224-specific responses, B6 mice were inoculated with 45 nmol Th-P2-CA, the unlipidated construct, or infected i.n. with HKx31. D\(^{\text{b}}\)PA224-specific responses could be measured in both the spleen and lung on day 9 after Th-P2-CA administration, although the numbers were significantly lower in mice that had received the lipopeptide vaccine compared with those observed after virus infection (Fig. 1A, p < 0.001, and B, p < 0.0004, respectively). Although the magnitude of D\(^{\text{b}}\)PA224-specific memory CTL found in the spleen was greater in mice that had resolved virus infection compared with lipopeptide-vaccinated mice (Fig. 1C, p < 0.015), there were equivalent numbers in the lung (Fig. 1D). Importantly, the difference in splenic memory CTL numbers between these groups (2-fold) at day 31 (Fig. 1C) after infection did not reflect the difference in effector CTL numbers at day 9 (10-fold; Fig. 1A). At each time point, the number of D\(^{\text{b}}\)PA224-specific CTL was always greater for the lipidated peptide vaccine construct compared with the unlipidated construct (Fig. 1).

**Administration of Th-P2-CA lipopeptide results in decreased viral lung titers after challenge**

To examine whether Th-P2-CA vaccine would provide protection from influenza A virus, naive mice and mice previously primed i.n. with 45 nmol Th-P2-CA or PR8 virus were infected with A/HKx31 and viral lung titers were determined on days 2, 6, and 8 after infection (Table I). The HKx31 virus used had a mutation introduced into the NP\(_{366}\) peptide (position 5 Asn-Gln mutation) abrogating peptide binding to H2-D\(^{\text{b}}\). As a consequence, the major D\(^{\text{b}}\)NP\(_{366}\)-specific CTL response is not present (27). Viral growth peaked to similar levels 2 days postinfection in both primed and naive mice. By day 6, both the PR8- and Th-P2-CA-primed mice demonstrated ∼15- to 20-fold lower lung viral titers compared with unprimed mice (Table I). This result contrasts previous reports describing poor protection after vaccination with PA224 peptide-pulsed DCs (31, 32). Despite the absence of a large D\(^{\text{b}}\)NP\(_{366}\)-response, the PR8 primed mice had completely cleared virus by day 8 postinfection and two of three of the lipopeptide mice had also completely cleared virus. In contrast, two of three of the naive mice had significantly higher levels of virus still present in the lungs. These results demonstrate that cellular immunity generated after D\(^{\text{b}}\)PA224-specific lipopeptide vaccination is capable of limiting viral replication after challenge.

The magnitude of IFN-γ-D\(^{\text{b}}\)PA224-specific CTL responses was equivalent in both the spleen (Fig. 2A) and lung (Fig. 2B) of mice previously primed with either the lipopeptide vaccine or PR8 virus.

### Table I. Pulmonary viral titers after viral challenge of lipopeptide-inoculated mice

<table>
<thead>
<tr>
<th>Day Postinfection</th>
<th>PR8</th>
<th>Th-P2-CA</th>
<th>PBS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>2.3 ± 10⁶</td>
<td>2.0 ± 10⁶</td>
<td>3.3 ± 10⁴</td>
</tr>
<tr>
<td>6</td>
<td>1.3 ± 10⁴</td>
<td>1.8 ± 10⁴</td>
<td>3.8 ± 10³</td>
</tr>
<tr>
<td>8</td>
<td>0.0 ± 0.0</td>
<td>1.2 ± 10⁴</td>
<td>4.0 ± 10³</td>
</tr>
</tbody>
</table>

*Naive B6 mice were either primed with 1.5 × 10⁷ PFU PR8 virus i.p., inoculated with 45 nmol Th-P2-CA or 30 \(\mu\)l of PBS i.n. After 6 wk to allow establishment of memory, mice were infected with 10⁷ PFU HKx31 NP NSQ virus i.n. On days 2, 6, and 8 postinfection, lungs were assayed for infectious virus by plaque assay. The data show the mean number of PFU per lung. SD is for three mice per group for days 2 and 8, and five mice per group for day 6.
A comparison of memory D\textsuperscript{A}PA\textsubscript{224}-specific CTL recalled with either lipopeptide or virus inoculation. PR8-primed B6 mice were given either 10\textsuperscript{5} PFU HKx31 (A and B), or 45 nmol Th-P2C-PA (C and D) i.n. Tissues were harvested 8 days later and lymphocytes were stimulated with 1 \textmu M PA\textsubscript{224} peptide for 5 h in the presence of brefeldin A, stained with anti-CD8\textalpha-PerCP, Cy5.5, fixed, permeabilized, and stained for intracellular IFN-\gamma and TNF-\alpha. The numbers shown on the FACS profiles are the percentage of TNF-\alpha\textsuperscript{+} of IFN-\gamma\textsuperscript{+} CD\textsuperscript{8}\textsuperscript{+} T cells. Shown are representative cytokine profiles for the spleen (A and C) and lung (B and D).

There was no difference in the proportion of IFN-\gamma\textsuperscript{+} D\textsuperscript{A}PA\textsubscript{224}-specific CTL producing either TNF-\alpha (Fig. 2, C and D) or IL-2 (Fig. 2, E and F). Importantly, vaccination with the unlipidated PA\textsubscript{224} construct did not result in establishment of robust memory as the recall response was significantly diminished in both the spleen (Fig. 2A, p < 0.001) and lung (Fig. 2B, p < 0.05). Therefore, D\textsuperscript{A}PA\textsubscript{224}-specific CTL primed with lipopeptide exhibited protective responses that were functionally equivalent to CTL primed with virus infection.

Comparison of memory D\textsuperscript{A}PA\textsubscript{224}-specific CTL recalled after either lipopeptide or influenza A virus infection

Given the magnitude of immunity was equivalent for lipopeptide- and virus-primed memory CTL recalled with virus infection, it was of interest to determine whether CTL primed with lipopeptide were functionally equivalent to those generated after virus infection. However, the numbers of D\textsuperscript{A}PA\textsubscript{224}-specific CTL elicited after primary lipopeptide administration were too low for reasonable assessment of the response using conventional assays (16). Therefore, the qualitative characteristics of virus-primed memory cells, recalled with either Th-P2C-PA lipopeptide or heterologous virus challenge, were compared. Lymphocytes were isolated from either the spleen or lung and intracellular cytokine staining performed to assay both the magnitude and functional quality of recalled D\textsuperscript{A}PA\textsubscript{224}-specific CTL. As previously described (22), cytokine production by both lipopeptide- and virus-recalled D\textsuperscript{A}PA\textsubscript{224}-specific CTL followed a hierarchy with TNF-\alpha producers a subset of the IFN-\gamma producers (Fig. 3). Although administration of the Th-P2C-PA lipopeptide vaccine resulted in a smaller recall response in the spleen when compared with virus challenge (Fig. 4A, p < 0.001), the recall response was equivalent in the lung (Fig. 4B).

Importantly, the Pam2Cys moiety was required for efficient expansion of memory CTL as the number of D\textsuperscript{A}PA\textsubscript{224}-specific CTL recalled with the unlipidated construct was significantly lower than either A/HKx31 or lipidated Th-P2C-PA challenge (Fig. 4, A and B).

Analysis of the proportion of IFN-\gamma\textsuperscript{+} CTL producing either TNF-\alpha\textsuperscript{+} or TNF-\alpha/IL-2 has previously been used to determine qualitative differences in CTL populations after influenza A virus infection (22, 25). To determine whether lipopeptide administration altered the capacity of D\textsuperscript{A}PA\textsubscript{224}-specific CTL to produce cytokine, the proportion of IFN-\gamma\textsuperscript{+} D\textsuperscript{A}PA\textsubscript{224}-specific CTL producing multiple cytokines was determined. A significantly lower proportion of TNF-\alpha\textsuperscript{+} D\textsuperscript{A}PA\textsubscript{224}-specific CTL from both spleen (Fig. 4C) and lung (Fig. 4D) was observed in lipopeptide-challenged mice compared with those infected with heterologous virus. This apparently lower quality cytokine response was also reflected in a significantly lower proportion of IL-2\textsuperscript{+} CTL (Fig. 4, E and F). Although CTL recalled with either the Th-P2C-PA or Th-PA constructs resulted in a lower proportion of TNF-\alpha producers compared with virus challenge (Fig. 4C, D), it was only Th-P2C-PA administration that resulted in a lower proportion of IL-2 producers (Fig. 4, E and F). The high proportion of IL-2\textsuperscript{+} CTL found in the Th-PA and PR8-PBS group reflects the lack of expansion of pre-existing memory CTL that typically demonstrate maturation to multiple cytokine production (22).

Analysis of the amount of cytokine produced, determined by measuring the mean fluorescence intensity (MFI) of staining, demonstrated that splenic and lung D\textsuperscript{A}PA\textsubscript{224}-specific CTL recalled after Th-P2C-PA administration expressed lower levels of IFN-\gamma.
Comparison of tetramer dissociation and TCR repertoire diversity of D\textsuperscript{7}PA\textsubscript{224}-specific CTL recalled by either virus or lipopeptide challenge

Previous analysis of influenza A virus-specific CTL populations demonstrated that an increased capacity to produce multiple cytokines correlated with a higher TCR-binding avidity (23, 25). Given the decreased capacity of lipopeptide-induced D\textsuperscript{7}PA\textsubscript{224}-specific CTL to produce IFN-\(\gamma\) and TNF-\(\alpha\), the TCR-binding avidity was analyzed (Fig. 6). Despite the observed differences in cytokine production (Figs. 4 and 5), there was no difference in the tetramer dissociation kinetics of D\textsuperscript{7}PA\textsubscript{224}-specific CTL after virus or lipopeptide challenge. This was the case for both splenic (Fig. 6A) and lung (Fig. 6B) CTL populations.

![Figure 4](image-url) Mode of challenge impacts D\textsuperscript{7}PA\textsubscript{224}-specific CTL cytokine profiles. PR8-primed B6 mice were given 10\(^5\) PFU x31, 45 nmol Th-L-PA, 45 nmol Th-PA, or 30 \(\mu\)l of PBS i.n. Lymphocytes were isolated from the spleen and lung and stimulated with PA224 peptide as described in Fig. 2. Samples were then stained simultaneously for IFN-\(\gamma\), TNF-\(\alpha\), and IL-2. Results for D\textsuperscript{7}PA\textsubscript{224}-specific CTL derived from spleen (A, C, and E) and lung (B, D, and F) are shown. The number of CD8\(^+\) D\textsuperscript{7}PA\textsubscript{224}-specific CTL are shown in A and B. The proportion of IFN-\(\gamma\)-coexpressing TNF (C and D) and IL-2 (E and F) are shown. Data show mean ± SD of four mice and are representative of three experiments. Significance was determined using a Student t test (*, \(p < 0.05\); **, \(p < 0.01\) with respect to samples from PR8/HKx31).

![Figure 5](image-url) D\textsuperscript{7}PA\textsubscript{224}-specific CTL recalled with lipopeptide exhibit lower levels of IFN-\(\gamma\) and TNF-\(\alpha\), but not IL-2. Using the samples described in Fig. 4, the relative abundance of each cytokine was measured by the MFI of staining. Results show the average MFI of IFN-\(\gamma\) (A and B), TNF-\(\alpha\) (C and D), and IL-2 (E and F) staining for the D\textsuperscript{7}PA\textsubscript{224}-specific CTL isolated from either spleen (A, C, and E) or lung (B, D, and F). Shown is the mean ± SD (\(n = 4\)), representative of three experiments. Significance was determined using a Student t test (*, \(p < 0.05\); **, \(p < 0.01\) with respect to samples from PR8/HKx31).

![Figure 6](image-url) Comparison of TCR avidity by tetramer dissociation. A tetramer dissociation assay was used to compare the TCR avidity of D\textsuperscript{7}PA\textsubscript{224}-specific CD8\(^+\) cells recovered from the spleens and lungs of PR8-primed mice inoculated with 10\(^6\) PFU HKx31 (*) or 45 nmol Th-P2C-PA( ), 45 nmol Th-PA (A) or PBS ( ). Cells were stained with D\textsuperscript{7}PA\textsubscript{224} tetramer and then incubated at 37\(^\circ\)C in the presence of anti-H2Db Ab to prevent rebinding of dissociated tetramer. Tetramer staining of CD8\(^+\) cells was evaluated at the indicated time points. Results are plotted as the natural log of the normalized fluorescence vs time for the spleen (A) and lung (B). Shown is the mean ± SD (\(n = 3\)), representative of three experiments.
of the TRBV29 TCR repertoire diversity of DbPA224-specific CTL and data not shown). Furthermore, there was no difference in TRBV29 lipopeptide demonstrated preferred selection of the TRBV29 gene-specific CTL isolated from mice that received either virus infection or 2/3 (formerly V.

Interestingly, there was an increase in the use of the TRBV, TRBJ, and CDR3β length was analyzed for DPA224-specific CTL isolated from naive mice either infected with AHKx31 or vaccinated i.n. with the lipopeptide (Table II and data not shown). DPA224-specific CTL isolated from mice that received either virus infection or lipopeptide demonstrated preferred selection of the TRBV29 gene-segment (Table II and data not shown). Lipopeptide-induced TRBV29 DPA224-specific CTL maintained the preferred usage of the TRBJ2-7 gene segment and a CDR3β length of 6-7 aa (Table II and data not shown). Furthermore, there was no difference in the TRBV29 TCR repertoire diversity of DPA224-specific CTL recalled with either lipopeptide or virus challenge (Table II). Interestingly, there was an increase in the use of the TRBV13-2/3 (formerly Vβ8.1/2) gene segment by lipopeptide induced DPA224-specific CTL (data not shown). In summary, lipopeptide administration results in a broader repertoire with expanded CTL using a normally subdominant TRBV13-2/3 gene segment without impacting on repertoire diversity of preferred TRBV29 TCR gene-segment usage.

### Discussion

This study compared the quality of cellular immunity generated after lipopeptide vaccination to that observed after virus infection. The number of resident memory CTL in the lungs was equivalent to that observed after virus infection despite greater numbers of DPA224-specific CTL in infected mice at the acute time point. This may reflect the fact that the lipopeptide construct contains a Pam2Cys moiety and has the capacity to activate DCs, presumably via TLR2 activation, with minimal inflammation (12, 14, 15). Vaccination with activated, peptide-loaded DCs resulted in rapid establishment of Listeria monocytogenes-specific cellular immune memory and the resulting secondary responses were equivalent to those observed after infection (9). Importantly, the generation of memory CTL was accelerated in DC-vaccinated animals due to the lack of inflammation (9, 33). It is therefore tempting to speculate that lipopeptide vaccines may, in fact, result in accelerated memory CTL formation without the need for large expansion of effector CTL. This notion warrants further investigation because an important implication is that vaccine regimens could be administered more rapidly, helping improve vaccine coverage.

Of particular interest was comparison of qualitative measurements of DPA224-specific CTL when recalled with either the Th-P2C-PA lipopeptide or virus infection. Importantly, there were distinct functional differences in the capacity of Th-P2C-PA recalled CTL to produce IFN-γ and TNF-α, but not IL-2, compared with virus-recalled CTL. Interestingly, there was no functional difference in memory CTL that were established after either primary infection or lipopeptide vaccination, then recalled after heterologous viral challenge. Therefore, this suggests that it is the mode of challenge that alters functional outcome of recalled CTL, rather than the mode of priming used to establish CTL memory. Why would the mode of Ag challenge alter the functionality of recalled CTL? Given the repertoires and magnitudes of recalled responses in the lung were similar for virus- and lipopeptide-recalled CTL, the observed functional differences most likely reflect differential modulation of cytokine production rather than differential recruitment of memory CTL. This modulation may be influenced by regulatory mechanisms controlling the biochemical modification of gene-specific promoters (34–36). Such modifications can be influenced by the inflammatory milieu induced after immune challenge (34). Given lipopeptide administration may not induce as much inflammation as viral infection, it is tempting to speculate that perhaps lipopeptides may not induce the appropriate biochemical modifications that would otherwise result in full effector function. If this was indeed the mechanism, it might also explain why there was no observed difference in cytokine production when lipopeptide- and virus-primed memory CTL are recalled with heterologous virus challenge. However, this is notion is speculative and warrants further investigation.

A more simple explanation may relate to differences in the kinetics of CTL responses after recall with either lipopeptide vs virus infection. We have previously demonstrated that the capacity of influenza A virus-specific CTL to produce multiple cytokines varies over the course of both primary and recall responses to infection (22). Therefore, it is possible that earlier analysis of CTL recalled with lipopeptide may have revealed CTL with similar cytokine profiles to that observed for CTL recalled after virus infection. In summary, while the data suggest that presentation of epitopes via lipopolysaccharides has the capacity to alter effector T cell function, it does not impact on the establishment of robust cellular immunity.

Interestingly, while the proportion of IL-2 producers was lower in lipopeptide-recalled CTL populations, the level of IL-2 production (measured by MFI) was not altered compared with both IFN-γ and TNF-α. Previous reports have associated the level of cytokine production with CTL TCR avidity for a given pMHC (22, 25). There was no difference in the TCR avidity of DPA224-specific CTL recalled with either lipopeptide or virus. Therefore, measurement of immune correlates such as TCR avidity and the level of IL-2 production may provide novel measures for evaluating cellular immunity induced after vaccination.

Ag-specific TCR repertoire diversity has recently emerged as an important facet of cellular responses to pathogens (37). There is growing evidence that increased TCR diversity is associated with protection from virus infection (38), minimizing CTL escape during persistent infections (39), and maximizing the potential for cross-reactive CTL responses to different pathogens (40). Given pMHC structures can impact on TCR diversity (41), selection of vaccine peptide Ags that elicit the appropriate TCR repertoire diversity is likely to be critical for vaccine efficacy. Interestingly, the DPA224-specific CTL recalled with lipopeptide demonstrated a broader TCR repertoire compared with those recalled with heterologous virus challenge. Normally after influenza A virus infection, TRBV13-2/3 usage by DPA224-specific CTL is subdominant to the more prevalent TRBV29 gene segment (42). Given the low CTL numbers induced after primary administration of lipopeptide, it is difficult to ascertain whether a similar increase in TRBV13-2/3
usage is observed (data not shown). It will be of interest to determine whether similar expansions of subdominant TCR biases are observed for other lipopeptide constructs (38, 39).

There was no difference in the TCR repertoire diversity within the prominent TRBV29 TCR gene segment of DPA224-specific CTL recalled with either lipopeptide or virus. The same finding was made when comparing the TCR repertoire diversity of DPA224-specific CTL when the PA224 epitope was ectopically expressed in the neuraminidase of influenza A virus (23). Together, this supports the notion that structural characteristics of a given pMHC antigenic complex, rather than epitope context, determines TCR repertoire diversity (37, 41). Overall, given recent positive associations between increased breadth of CTL responses and control of virus infection, lipopeptides may provide robust cellular immunity due to increased breadth of TCR repertoire diversity not typically observed after infection.

Administration of the Th-P2C-PA lipopeptide resulted in accelerated viral clearance correlating with increased DPA224-specific CTL responses. This is in contrast to studies demonstrating that priming of DPA224-specific CTL using DC vaccination resulted in delayed viral clearance compared with that observed after infection of naive mice (31, 32). It was suggested that delayed viral clearance was a consequence of poor presentation of the PA224 peptide presentation on lung epithelial cells at the site of infection (32). However, the protection observed after lipopeptide vaccination supports the notion that DPA224-specific CTL are important in protection from influenza A virus infection (27). Importantly, recent evidence suggests that under appropriate inflammatory conditions, PA224 presentation can be demonstrated for nonprofessional APCs (43).

One possible explanation for the observed discrepancy is that the DPA224-specific CTL response, induced after the DC priming used by Crowe et al. (31, 32), was in some way of a lower quality than that observed after infection. Use of DCs previously activated with activating agents such as CpG or LPS have been demonstrated to induce similar levels of CTL responses to that observed after L. monocytogenes infection (9). Treatment of DCs with synthetic lipopeptide constructs, like that used in this study, result in high levels of activation, similar to those observed after CpG oligonucleotides or LPS treatment (12, 14, 15). The priming strategy used by Crowe et al. (31), however, used DCs that were not activated by pretreatment with these agents. Therefore, it is conceivable that differences in DC activation during priming resulted in different outcomes to infection. This highlights the fact that measurement of cell surface markers, such as increased expression of MHC class II (31), as a surrogate for DC activation may not suffice as measure of for priming of CTL responses. Another possible explanation for the discrepant results is the different routes of vaccination used between the studies. The lipopeptide was administered intranasally where it resulted in the establishment of long-lived lung-resident memory CTL (31). In the studies by Crowe and colleagues, the DCs were given i.v. and it is not clear whether lung-resident, memory DPA224-specific CTL were generated. The presence of resident, long-lived CTL in peripheral tissues after resolution of infection has been described for a number of pathogens (44–47) and are thought to play an important role in initial control of secondary infection (45, 48). Respiratory lipopeptide administration resulted in similar levels of resident DPA224-specific memory CTL in the lung tissue to that seen after virus infection. Therefore, it is possible that the protective effect offered by lipopeptide vaccination was due, in part, to establishment of memory DPA224-specific CTL in the appropriate anatomical location before secondary challenge.

This study has, for the first time, evaluated qualitative aspects of the CTL response induced after lipopeptide administration. Importantly, our results have highlighted how appropriate activation of DCs and route of vaccination are important considerations when designing and evaluating novel vaccine strategies for inducing cellular immunity. Furthermore, lipopeptides also provide an important tool for dissecting the factors that contribute to robust cellular immunity such as TCR avidity, TCR repertoire diversity, and function. Such tools are essential to better identify the measurements of CTL function that correlate with protective immunity.

Acknowledgments

We thank Drs. Nicole La Gruta and Lorena Brown for critical review and discussion, and Dina Stockwell for excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

References

LIPOPEPTIDE VACCINATION AGAINST INFLUENZA A VIRUS


