Addition of a Prominent Epitope Affects Influenza A Virus-Specific CD8+ T Cell Immunodominance Hierarchies When Antigen Is Limiting

Misty Rayna Jenkins, Richard Webby, Peter C. Doherty and Stephen J. Turner

*J Immunol* 2006; 177:2917-2925; doi: 10.4049/jimmunol.177.5.2917
http://www.jimmunol.org/content/177/5/2917

**References**

This article cites 36 articles, 24 of which you can access for free at:
http://www.jimmunol.org/content/177/5/2917.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
Addition of a Prominent Epitope Affects Influenza A Virus-Specific CD8+ T Cell Immunodominance Hierarchies When Antigen Is Limiting

Misty Rayna Jenkins,* Richard Webby,† Peter C. Doherty,*‡ and Stephen J. Turner2*

A reverse genetics strategy was used to insert the OVA peptide (amino acid sequence SIINFEKL; OVA257–264) into the neuraminidase stalk of both the A/PR8 (H1N1) and A/HK×31 (H3N2) influenza A viruses. Initial characterization determined that KnOVA257 is presented on targets infected with PR8-OVA and HK-OVA without significantly altering D b nucleoprotein (NP)366 presentation. There were similar levels of KnOVA257 and D b NP366-specific CTL expansion following both primary and secondary intranasal challenge. Interestingly, while variable, the presence of the immunodominant KnOVA257-specific response resulted in diminished D a acidic polymerase224- and K b basic polymerase subunit 1703-, but not D b NP366-specific responses and didn’t alter endogenous influenza A virus-specific immunodominance hierarchies. However, challenging PR8-OVA-primed mice with HK-OVA via the i.p. route, and thereby limiting Ag dose, led to a reduction in the magnitude of all the influenza A virus-specific responses measured. A similar reduction in CTL response to native epitopes was also seen following primary respiratory HK-OVA infection of mice that received substantial numbers of KnOVA257-specific TCR transgenic T cells. Thus, during the course of infection, the generation of individual virus-specific CTL responses is independently regulated. However, in cases in which Ag is limiting, or high precursor frequency, the presence of immunodominant CTL responses can impact on the magnitude of other specific populations. Therefore, depending on both the size of the T cell precursor pool and the mode of Ag presentation, the addition of a major epitope can diminish the size of endogenous, influenza-specific CD8+ T cell responses, although never to the point that these are totally compromised. The Journal of Immunology, 2006, 177: 2921–2925.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received for publication December 14, 2005. Accepted for publication June 16, 2006.

Copyright © 2006 by The American Association of Immunologists, Inc.
response can immunodominate the D*PA224-specific response in the secondary response (22). However, it has been demonstrated that the primary D*PA224 response is not affected after transfer of D*Np366-specific CTL (22, 23). Furthermore, in the absence of a secondary D*Np366 response, the D*PA224-specific CTL response fails to compensate to any significant extent (9, 24). These studies imply that immunodominance by D*Np366 may in fact play a minor role in determining the magnitude of the D*PA224-specific response. However, concurrent examination of subdominant D*PB1-F262-70 T, K*PB1-103 T, and K*N2S114-specific responses showed an increase in magnitude in the absence of D*Np366- and D*PA224-specific responses (9, 24). The conclusion from these studies was that subdominant CTL responses compensate for loss of immunodominant responses after influenza A virus infection. Given the discrepancy in the literature, further studies are required to help determine the consequence of CTL immunodominance on Ag-specific hierarchies established after influenza A virus infection.

To further examine the potential for immunodominance in the influenza A-specific CTL response after infection, primary and secondary D*Np366, D*PA224, and K*PB1-703 CTL responses were analyzed after infection with an influenza A virus containing the immunogenic OVA peptide (OVA257; amino acid sequence SIINFEKL, presented by H2Kb) within the stalk of influenza virus neuraminidase (NA) protein. The presence of an immunodominant K*OVA257-specific CTL response resulted in a trend for diminished CTL responses to the native influenza A virus epitopes, although this result was variable. Interestingly, the presence of K*OVA257-specific CTL responses could reproducibly suppress the D*PA224- and K*PB1-703-specific CTL responses when Ag was limiting during a nonproductive infection. Moreover, transfer of OT-I TCR Tg CTL specific for the K*OVA257 epitope into naive mice resulted in diminished CTL responses to D*Np366-, D*PA224-, and K*PB1-703 after infection. Importantly, this effect could not be rescued for D*PA224- and K*PB1-103-specific CTL responses by increasing the level of epitope presentation. Overall, these data suggest that in cases in which either viral Ag is limiting, or the T cell precursor frequency for particular epitopes is high, CTL immunodominance of other specificities is apparent.

Materials and Methods

Mice and tissue sampling

Female C57BL/6J (B6, H-2b) Thy-1.2+ and Tg B6-OT-I Thy-1.1+ mice were bred in the animal facility at the Department of Microbiology and Immunology at University of Melbourne and were held under specific pathogen-free conditions. Naive mice at least 6 wk of age were lightly anesthetized by inhalation of (methoxyfluoro) pentane and infected intranasally (i.n.) with 10⁶ PFU of A/HK×31 strains of virus in 30 µl. Mice for secondary challenge experiments were primed by i.p. injection with 1.5 × 10⁷ PFU of the A/P88 viruses at least 6 wk previously. The A/HK×31 and A/P88 influenza A viruses differ in their surface hemagglutinin and NA, but share the PR8 internal proteins (NP, NS1, NS2, M, PA, PB1, and PB2). At the time of tissue sampling, mice were anesthetized and exsanguinated by section of the axillary artery. The inflammatory cell population obtained by bronchoalveolar lavage (BAL) was incubated on plastic for 1 h at 37°C to remove adherent macrophages (25). In one experiment, lymphocytes were isolated from perfused, minced lung tissue by collagenase digestion (19). Single-cell suspensions of spleen were enriched for CD8+ T cells by incubation on plastic tissue culture plates precoated with 200 µg/ml anti-IgG and anti-IgM Abs (Australian Laboratory Services) for 1 h at 37°C, 5% CO₂.

Generation and titration of recombinant viruses

PCR primers encoding for the OVA257-264 SIINFEKL peptide and part of the NA from A/HK×31 or A/P88 (sequences are available upon request) were used with NA-specific primers (26) to amplify the NA segment from either A/HK×31 or A/P88. The PCR products were digested with BanB I and ligated into pHW2000 (26). The recombinant A/HK×31 and A/P88 viruses (in this study referred to as HK-OVA and PR8-OVA, respectively) were rescued after transfection of mixed cultures of 293T and Madin-Darby canine kidney (MDCK) cells using the eight-plasmid reverse genetics system described by Hoffmann et al. (27). Recombinant influenza A viruses were grown at 33°C for 2 days in the allantoic cavity of 10-day embryonated hen eggs, then incubated at 4°C overnight before the allantoic fluid was harvested and clarified by centrifugation at 3000 × g for 10 min. The virus stocks used for infection were grown in eggs. Viral titers were determined from allantoic fluid (or lung homogenates from infected mice) by plaque assay on confluent MDCK cell monolayers (28).

Peptide stimulation and intracellular cytokine staining

Enriched spleen- and macrophage-depleted BAL lymphocyte populations were cultured for 5 h at 37°C in 96-well round-bottom plates at −0.5–2 × 10⁶ cells/well in complete RPMI 1640 containing 10% FCS, 10 U/ml human rIL-2, and 5 µg/ml GolgiPlug (BD Biosciences), with or without 1 µM Np366–374 (amino acid sequence ASNNENMETM), PA224–236 (amino acid sequence SSLENFRAYV), PB1703–711 (amino acid sequence SSSRPRVGI), or OVA257–264 (amino acid sequence SIINFEKL) peptides (Ausspep). The cells were then washed with PBS (containing 0.1% BSA and 0.02% sodium azide), stained with anti-mouse CD8a-PerCP-Cy5.5 (BD Pharmingen) for 30 min on ice, permeabilized by paraformaldehyde fixation using the BD Cytofix/Cytoperm Kit (BD Biosciences), and stained for intracellular cytokine production using anti-mouse IFN-γ FITC (clone XMG1.2; BD Pharmingen) and anti-TNF-α PE (clone M15-8D; BD Pharmingen). The lymphocytes were then washed and analyzed on a FACSCalibur, and analysis was performed using CellQuest Pro software (BD Biosciences). In each assay, any cytokine-positive cells isolated from wells with no peptide were subtracted from the percentage of cytokine-positive cells incubated with peptide to yield the final value.

CD8+ T cells lines and CTL assay

Splenocytes from naive B6 mice were resuspended (10⁶ cells/ml) in HBSS, incubated with 1 µM peptide for 1 h at 37°C (15), irradiated (3000 rad), washed twice in HBSS, and mixed (3 × 10⁵ cells) with equal numbers of splenocytes from B6 mice that had been infected i.n. 10 days previously with 10⁶ PFU of the HK virus. They were then resuspended in 40 ml of complete RPMI 1640 (10% FCS/penicillin-streptomycin/glutamine/5 × 10⁻⁵ M 2-ME) and cultured for 5 days at 37°C, 5% CO₂. After 5 days, a CTL assay was performed. The target EL4 cells used for the CTL assay were either infected with 10 multiplicity of infection of PR8-OVA or HK-OVA (1 h in 300 µl of HBSS, then 2 h in RPMI 1640) or pulsed with 1 µM peptide, then labeled with 300 µCi of 5¹Cr (Amersham Biosciences) for 1 h at 37°C. Effector T cells were serially diluted across a range of E:T ratios in 96 round-bottom plates. The percentage of specific lysis at 6 h was calculated as 100 × [Cr release from targets alone]–[Cr release from targets alone+10⁴ Cr release from targets with 1% Triton X-100] × 100/ [Cr release from targets alone]. The level of ⁵¹Cr release from targets alone did not exceed 10% of the total ⁵¹Cr release from targets with 1% Triton X-100.

Adaptive transfer experiments

Naïve splenocytes were obtained from B6-OT-I-Cd45.1+ mice (29) and enriched for CD8+ T cells by depletion after incubation on plastic tissue culture plates precoated with 200 µg/ml anti-IgG and anti-IgM Abs for 1 h at 37°C, 5% CO₂, before staining 1 × 10⁶ cells/ml with 5 µM fluorescent dye CFSE (Sigma-Aldrich), according to Turner et al. (23). The labeled cells were transferred i.v. into naive B6 Cd45.1+ mice. Before transfer, the CD8+ T cell population displayed the naïve Cd69lowCd262highCd44high phenotype (data not shown). Recipient mice were infected i.n. with 1 × 10⁷ PFU of HK-OVA 24 h after cell transfer and sampled after an additional 8 days.

Results

Presentation and recognition of the K*OVA257 epitope

A reverse genetics strategy (27) was used to insert the OVA257-264 peptide (SIINFEKL) presented by H2-Kb into the NA stalk of the A/P88 (H1N1) and A/HK×31 (H3N2) influenza A viruses. The ability of peptide-stimulated CTL lines to kill either PR8-OVA or HK-OVA virus (data not shown)-infected EL4 (D*Kb) cells established that infection with either virus induced the expression of the K*OVA257 epitope (Δ; Fig. 1A). The level of K*OVA257-specific ⁵¹Cr release was broadly equivalent to the killing activity found for the native D*Np366 after PR8 infection (compare Δ; Fig. 1, A and B). Furthermore, the presence of OVA257 in the viral NA neither
changed the sensitivity of the infected EL4 cells to D\textsuperscript{\texttextit{\textalpha}}NP366\textsuperscript{-specific CTL lysis (Fig. 1B, compare \textbullet{} and \textDelta{}), nor modified the apparent lack of expression of the D\textsuperscript{\textbeta}PA224 epitope (Fig. 1C, compare \textbullet{} and \textDelta{}). Both the PR8-OVA and HK-OVA viruses thus induce the expression of K\textalpha{}OVA\textsubscript{257} without obviously altering the normal MHC class I-restricted presentation profile characteristic of infection with these influenza A viruses.

Reduced virus titers in the lung after challenge with HK-OVA virus

Previous reports have demonstrated that insertion of short peptides into the NA of influenza A virus strain, A/WSN/33, can result in attenuation of in vivo virus growth after infection (30, 31). To determine whether the presence of the OVA\textsubscript{257} epitope in the NA of HK-OVA resulted in attenuation of the virus, mice were infected i.n. with equivalent doses containing 10\textsuperscript{4} PFU of either the HK or HK-OVA viruses. Virus lung titers were determined by plaque assay at intervals after respiratory exposure. At all time points assayed, the HK-OVA viral titers were significantly lower than in mice given an equivalent dose of HK virus (p < 0.01 at day 3 postinfection; p < 0.03 at days 5 and 7 postinfection) (Fig. 2A). The lower HK-OVA viral titer at day 3 was unlikely to be due to a K\textalpha{}OVA\textsubscript{257}-specific response, as infection of MHC-mismatched BALB/c mice (H-2\textsuperscript{\textbeta}\textsuperscript{-}) with the HK-OVA virus also resulted in lower peak viral titers at day 3 compared with the wild-type HK virus (Fig. 2B). Interestingly, despite the HK-OVA virus growing to a lower peak viral titer, it was cleared at the same time as the wild-type HK virus (Fig. 2A). Therefore, in agreement with previous studies (30, 31), it seems that insertion of the OVA\textsubscript{257} peptide into NA resulted in attenuated viral growth in vivo. Such attenuation may have relevance for i.n. infection where productive infection by the HK-OVA virus may result in less Ag, and therefore not induce robust CTL responses.

Effect of K\textalpha{}OVA\textsubscript{257} on the influenza A virus-specific CTL response following primary infection

Given the observed attenuation of the HK-OVA virus, it was important to determine whether infection with HK-OVA would result in a decreased magnitude of CTL responses compared with HK infection. To determine both the impact of the attenuation and the impact of OVA\textsubscript{257} presentation on the size of responses directed toward D\textsuperscript{\textbeta}NP366, D\textsuperscript{\textalpha}PA224, and K\textsuperscript{\textbeta}PB1\textsubscript{703}. B6 mice were infected i.n. with 10\textsuperscript{4} PFU of the HK or HK-OVA viruses and sampled 10 days later. The lymphocytes obtained from spleen or by BAL were stimulated with 1 \mu M NP\textsubscript{366} (Fig. 3, A and E), PA\textsubscript{224} (Fig. 3, B and F), PB1\textsubscript{703} (Fig. 3, C and G), or OVA\textsubscript{257} (Fig. 3, D and H) peptides in the presence of brefeldin A, then stained subsequently with IFN-\gamma and TNF-\alpha-specific mAbs. Representative staining profiles are shown for the spleen (Fig. 3, A–H). In agreement with previous studies (16, 17), the majority of D\textsuperscript{\textbeta}PA224-specific CTL are positive for both IFN-\gamma and TNF-\alpha after peptide stimulation (Fig. 3, B and F), while D\textsuperscript{\textalpha}NP366-specific/TNF-\alpha\textsuperscript{-} CTL are only a subset of the IFN-\gamma\textsuperscript{+} CTL (Fig. 3, A and E). The cytokine profile of K\textalpha{}OVA\textsubscript{257}-specific CTL (Fig. 3H) was more similar to that of D\textsuperscript{\textalpha}NP366-specific CTL with TNF-\alpha\textsuperscript{-} CTL, a subset of the IFN-\gamma\textsuperscript{+} CTL (compare Fig. 3, E and H). Importantly, there was no evidence that OVA from egg allantoic fluid used to grow the virus stocks induces a K\textalpha{}OVA\textsubscript{257}-specific response when the virus is administered i.n. (Fig. 3D).

Quantitative analysis of the virus-specific CD8\textsuperscript{+} IFN-\gamma\textsuperscript{+} populations generated following primary challenge showed that concurrent expansion of the CD8\textsuperscript{+} K\textalpha{}OVA\textsubscript{257}\textsuperscript{+} specific set did not significantly diminish the size of the D\textsuperscript{\textbeta}NP366\textsuperscript{-} CTL response recovered from the spleen (Fig. 3I) or the infected lung (Fig. 3K). There was a trend for diminished D\textsuperscript{\textalpha}PA224- and K\textsuperscript{\textbeta}PB1\textsubscript{703}-specific responses in the spleen and lungs of mice that were infected with HK-OVA (Fig. 3, I and K). Although these diminished responses were variable and not significantly different in the spleen, in the lung there were 50% fewer K\textsuperscript{\textbeta}PB1\textsubscript{703}-specific T cells following HK-OVA infection compared with wild-type virus infection (Fig. 3K; p < 0.05). Importantly, the responses to D\textsuperscript{\textbeta}NP366 and K\textalpha{}OVA\textsubscript{257} were essentially comparable in magnitude (Fig. 3, I and K). By this criterion, K\textalpha{}OVA\textsubscript{257} can be regarded as a prominent epitope in the primary response to the HK-OVA virus. Summing the values for the four populations of CD8\textsuperscript{+} T cells indicated that, despite the apparent attenuation of HK-OVA, the total numbers of responders in either the BAL or spleen were, in fact, similar after infection with either HK or HK-OVA (Fig. 3, J and L). This is most likely due to the presence of the K\textalpha{}OVA\textsubscript{257}-specific response making up for the diminished D\textsuperscript{\textalpha}PA224 and K\textsuperscript{\textbeta}PB1\textsubscript{703} responses.
The consequences of OVA<sub>257</sub> expression for secondary challenge were next analyzed using mice that were primed i.p. with PR8-OVA and challenged i.n. 6 wk later with either HK- or HK-OVA (E–H). CD<sup>8</sup> T cells were stimulated with NP<sub>366</sub> (A–D) or HK-OVA (E–H) viruses, and the production of IFN-γ and TNF-α was determined for each of the four peptides. Due to the low frequency, the number of KbPB1703-specific CTL is represented by the inset of I and K. The average numbers of memory OT-I cells present in HK-infected mice are 71,981 ± 20,976 and 1,360 ± 228 in the spleen and BAL, respectively. Each data point shows the mean (n = 5) ± SD. These numbers were summed to give the total in the spleen (J) and BAL (L). Statistical significance was determined using an unpaired two-tailed Student’s t test (*, p < 0.05).

Effect of KbOVA<sub>257</sub> on the influenza A virus-specific CTL response following secondary infection

The consequences of OVA<sub>257</sub> expression for secondary challenge were next analyzed using mice that were primed i.p. with PR8-OVA, then given either the HK (Fig. 4, A–D) or HK-OVA (Fig. 4, E–H) viruses i.n. 6 wk later. Lymphocytes from both the spleen and BAL were stimulated with the various peptides, and the production of IFN-γ and TNF-α was determined. Representative staining profiles are shown for the spleen (Fig. 4, A–H). After challenge with HK-OVA, the proportion of CD<sup>8</sup> T cells specific for either KbOVA<sub>257</sub> or D<sup>NP</sup><sub>366</sub> was similar (Fig. 4, compare E and H). Furthermore, the proportion of TNF-α<sup>+</sup> of IFN-γ<sup>+</sup> KbOVA<sub>257</sub>-specific CTL was again similar to that observed for the D<sup>NP</sup><sub>366</sub>-specific set as seen after primary infection (Fig. 4, A, E, and H). Memory KbOVA<sub>257</sub>-specific CTL observed after HK challenge were largely IFN-γ<sup>+</sup>/TNF-α<sup>+</sup> (Fig. 4D), as has been described for other influenza-specific memory CTL populations (17). Memory KbOVA<sub>257</sub>-specific CTL could be found in the spleens and lungs of HK-infected animals, but showed no evidence of clonal expansion (Fig. 4, I and K, and data not shown), supporting the notion that specific Ag is required for expansion of memory CTL (23, 32). As in the primary response, there was a trend for...
diminished D\(^{8}\)PA\(_{224}\)- and K\(^{b}\)PB\(_{1703}\)-specific CTL responses in the presence of an immunodominant K\(^{b}\)OVA\(_{257}\) response, although these changes were not significant (Fig. 4, I and K). Although the presence of a large recall K\(^{b}\)OVA\(_{257}\) response resulted in a trend toward a greater total response in the spleen and infected lung, compared with HK secondary challenge, this difference was also not significant (Fig. 4, J and L). Overall, the pattern following both primary and secondary challenge reflected that the presence of the prominent secondary K\(^{b}\)OVA\(_{257}\) response caused no substantial change in the total magnitude of the CD\(^{8}\) T cell response.

Nonproductive challenge diminishes the native secondary CTL response

The earlier results suggested that the presence of the immunodominant OVA\(_{257}\)-specific CTL response resulted in diminished D\(^{8}\)PA\(_{224}\)- and K\(^{b}\)PB\(_{1703}\)-specific responses, although these results demonstrated a degree of variability. Importantly, despite the in vivo attenuation of the HK-OVA virus, the total specific CTL response was similar for both HK and HK-OVA viruses. Therefore, over the course of a replicative infection, enough Ag may be present for the optimal expansion of influenza A virus-specific population, reducing the impact of the OVA\(_{257}\)-specific response.

It is possible that the immunodominance of K\(^{b}\)OVA\(_{257}\) might be more obvious if viral dose, and therefore Ag load, was equalized for both viruses. Intrapneural infection with influenza A virus does not result in the production of mature virus due to the lack of a tissue-specific enzymatic cleavage of hemagglutinin required for replicative infection. Therefore, i.p. challenge results in nonreplicative infection, and consequently, the effective Ag dose following influenza virus challenge by a nonrespiratory route may be considered a direct reflection of the amount of virus (whether infectious or defective) in the input inoculum. Mice were primed i.p. with PR8-OVA, rested for 6 wk, then challenged i.p. with 1.5 \times 10^7 PFU of HK or HK-OVA (Fig. 5). In mice given HK-OVA, the magnitude of CD\(^{8}\)D\(^{8}\)NP\(_{366}\)-, CD\(^{8}\)D\(^{8}\)PA\(_{224}\)-, and CD\(^{8}\)K\(^{b}\)PB\(_{1703}\)-specific responses was significantly diminished when compared with mice given the HK-OVA virus (Fig. 5, A and B).

Previous experiments demonstrated that the D\(^{8}\)NP\(_{366}\)- and K\(^{b}\)OVA\(_{257}\)-specific CTL responses were codominant after i.n. challenge (Fig. 4). After i.p. priming, where there is no replicative infection and therefore limiting Ag, the immunodominance hierarchy was D\(^{8}\)NP\(_{366}\)\(>\)K\(^{b}\)OVA\(_{257}\) (Fig. 5B). Interestingly, the D\(^{8}\)NP\(_{366}\)-specific response was lower in magnitude after HK-OVA challenge compared with wild-type HK challenge (Fig. 5B), suggesting that the presence of the K\(^{b}\)OVA\(_{257}\)-specific response was impacting both dominant and subdominant CTL responses. Although there was a trend for an overall decrease in the size of the total Ag-specific T cell population in the spleen comparing HK- and HK-OVA-infected mice (Fig. 5C), this was not statistically significant (\(p = 0.13\)).

Consequences of adding Tg OVA\(_{257}\)-specific T cells to the response

A key characteristic of CD\(^{8}\) T cell memory is the increased size of the responder population (33), which is generally considered to be at least 100-fold higher than the naive T cell precursor frequency (34, 35). The secondary challenge experiments shown to date (Figs. 4 and 5) analyzed the nature of the recall response for a spectrum of memory T cell populations expanding in the presence, or absence, of a primed K\(^{b}\)OVA\(_{257}\)-specific set. Importantly, the immunodominant OVA\(_{257}\)-specific response only reproducibly diminished the host response to influenza A virus determinants when Ag was limiting. The availability of the HK-OVA virus and congenic TCR Tg OT-I mice, specific for K\(^{b}\)OVA\(_{257}\), allows analysis of the naive response to influenza epitopes when a naive TCR Tg population is both present and stimulated during the course of respiratory infection.

Conventional, naive Ly-5.2\(^{-}\)CD45.1\(^{-}\), B6 mice were injected with 10\(^{6}\) naive (CD62L\(^{hi}\)CD44\(^{low}\)), CFSE-labeled, Ly-5.1\(^{-}\}CD45.1\(^{+}\) OT-I T cells. Half of these mice were left unchallenged, while the remainder (along with OT-I B6 controls) were infected i.n. with 10\(^{6}\) PFU of HK-OVA 1 day after transfer. The substantial response to K\(^{b}\)OVA\(_{257}\) in the normal B6 mice was, of course, mediated by CD45.1\(^{-}\) cells (Fig. 6, A and B), while almost all of the K\(^{b}\)OVA\(_{257}\)-specific set detected in the spleen or lung of those given the OT-I cells originated from the transferred CD45.1\(^{+}\) population (Fig. 6, C and D, compare upper left and upper right panels).

Importantly, quantitative analysis of the Ag-specific T cell populations from spleen (Fig. 6E) and lung (Fig. 6F) showed that the concurrent expansion of the OT-I set significantly decreased the native (Ly-5.2\(^{-}\})CD45.1\(^{+}\) response to D\(^{8}\)PA\(_{224}\), K\(^{b}\)PB\(_{1703}\), and K\(^{b}\)OVA\(_{257}\), with the endogenous response to K\(^{b}\)PB\(_{1703}\) and K\(^{b}\)OVA\(_{257}\) (i.e., the two K\(^{b}\)-restricted epitopes) being the most affected. There was a trend for the D\(^{8}\)NP\(_{366}\)-specific response to be diminished in both the spleen and lung; however, this was not significantly different to the D\(^{8}\)NP\(_{366}\)-specific host response in the absence of OT-I cells (Fig. 6, E and F). The FACS profile for the lung populations recovered from these mice showed the presence of a substantial CD\(^{8}\) CD45.1\(^{+}\)IFN-\(\gamma\) set (Fig. 6D) that is present at much higher relative prevalence than in the spleen (Fig. 6C). This suggests there is recruitment of noncytokine-producing

**FIGURE 5.** Analysis of epitope-specific CTL after i.p. prime and challenge with PR8-OVA and HK-OVA viruses. Naive B6 mice were primed i.p. with PR8-OVA and challenged i.p. with HK [■] or HK-OVA [□] viruses 6 wk later. Epitope-specific CD8\(^{+}\) T cells from spleen were sampled 10 days later, stimulated with peptide, and analyzed for the production of intracellular IFN-\(\gamma\). The percentage (\(A\)) and number (\(B\)) of epitope-specific CD8\(^{+}\) T cells detected are shown as mean \pm SD (\(n = 5\)). The total counts (\(C\)) for peptide-specific CD8\(^{+}\) T cells (see legend for Fig. 2) were calculated for the five individual mice. Statistical significance was determined using an unpaired Student’s \(t\) test (\(*\, p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\)).
epitope-specific T cells that do not make cytokine to the site of inflammation. The total number of CD8+ IFN-γ+ Ag-specific T cells (including the Ly-5.1+CD45.1+ OT-I CTL) in the spleen (Fig. 7A) was unchanged. Interestingly, in the lungs of mice that received the OT-I T cells, there was a significant increase in the total number of IFN-γ+ CD8+ T cells (Fig. 7B).

Low numbers of transferred naive OT-I are capable of diminishing the host response to influenza A virus

Transfer of OT-I T cells can suppress not only the host OVA257-specific response, but also the host response to influenza A virus-specific determinants after HK-OVA infection (Fig. 6). In contrast, a similar analysis using lymphocytic choriomeningitis virus infection demonstrated that transfer of P14 TCR Tg T cells could diminish the host response to the same specificity, but left other specific responses unaffected (7). Moreover, immunodominance observed in this study was only apparent when large numbers of P14 TCR Tg cells were transferred. Therefore, the results observed may have been due to the large number of transferred OT-I TCR Tg cells.

To determine whether lower numbers of transferred OT-I cells would impact less on the host response to influenza A virus infection, naive CD45.1–B6 mice received either 1 × 10^6 (Fig. 8, Low OT-I) or 1 × 10^7 (Fig. 8, Hi OT-I) naive OT-I T cells. Groups of mice were infected with HK-OVA, as previously described, and the host (CD45.1+) CTL response was determined by IFN-γ production after stimulation with the appropriate peptide. In the spleen, the KbPB1703 and KbOVA257 host response was diminished after transfer of both high and low numbers of OT-I cells (Fig. 8, A and C). However, when the lymphocyte population isolated from the lung by BAL was analyzed, there was clear evidence of diminished host responses to D^bNP366, D^bPA224, KbPB1703, and KbOVA257 with transfer of both high and low numbers of OT-I cells (Fig. 8, B and D). Again, there was a hierarchy with the D^bNP366 response less affected by the transfer of OT-I T cells compared with the other responses (Fig. 8). Overall, this suggests that even low numbers of OT-I precursors can inhibit host responses after infection, particularly at the site of infection.
Coinfection with HK and HK-OVA does not rescue the OT-I immunodomination of the host response to influenza A virus infection

It has been proposed that immunodomination of the host responses by TCR Tg T cell transfer is a result of competition for Ag on APCs (7, 8). To determine whether OT-I domination of influenza A virus host responses could be overcome with more viral Ag, mice that had received 1 × 10^7 OT-I cells were infected with either HK, or HK-OVA, or coinfected with both HK and HK-OVA viruses (Fig. 9). The Ag-specific lymphocyte populations from the spleen (Fig. 9, A and C) and infected lungs (Fig. 9, B and D) were measured by IFN-γ production, as previously described.

In mice that were infected with HK-OVA, the host responses in the spleen and BAL to D^bPA224, K^bPB1703, and K^bOVA257 were significantly lower in the presence of OT-I cells than in mice that did not receive the OT-I cells (Figs. 8 and 9). Importantly, coinfection with HK and HK-OVA viruses did not restore the diminished host response to the K^bPB1703 and K^bOVA257 responses in either the spleen (Fig. 9, A and C) or BAL (Fig. 9, B and D). Coinfection with HK and HK-OVA viruses restored the D^bNP366 response and partially restored the D^bPA224 response in the infected lung (Fig. 9, B and D). Overall, this suggests that the OT-I cells are potent competitors not just for the endogenous responses of the same specificity, but also other concurrent responses whether Ag is more limited or not.

Discussion

We have demonstrated that introduction of the K^bOVA257 CTL epitope into influenza A virus results in an additional immunodominant CTL population after both the primary and secondary infection. The presence of the immunodominant K^bOVA257-specific response has little impact on the native immunodominant D^bNP366-specific CTL observed after either primary or secondary i.n. virus infection. In contrast, while variable, the presence of K^bOVA257-specific responses resulted in diminished D^bPA224- and K^bPB1703-specific primary and secondary responses.

A recent study demonstrated that the magnitude of the D^bNP366-specific response did not alter after primary infection with the influenza A virus strain, A/WSN, expressing the K^bOVA257 epitope (32). This is in agreement with the notion that immunodominance hierarchies established after primary infection are largely independent of each other (7). A similar conclusion was reached after analysis of the CTL responses generated after primary infection of mice with recombinant influenza A viruses lacking the immunodominant D^bNP366 and D^bPA224 epitopes. In this instance, there was little increase in the magnitude of subdominant CTL responses (9, 24), suggesting that the CTL responses generated were independent of each other. Although primary influenza A virus immunodominance hierarchies are established independently of each other, closer analysis of the secondary CTL response demonstrated that the magnitude of minor CTL responses increased significantly in the absence of the immunodominant D^bNP366 and D^bPA224-specific CTL responses. This suggests that immunodomination may play a role in determining CTL immunodominance hierarchies after influenza A virus infection (9), especially when there are large numbers of competing T cells.

It was possible to reproducibly decrease the magnitude of normal virus-specific CD8^+ T cell responses under conditions where either Ag was limiting, due to nonproductive replication, or where there was excess K^bOVA257-specific naïve CTL precursors. In this study, diminished responses to K^bPB1703, and to a lesser extent, D^bPA224 and D^bNP366 were observed. A possible explanation for this hierarchy might be that available Ag on APCs was more limiting for PB1703 and PA224 compared with the NP366. We have demonstrated previously that there is a greater abundance of NP and NA (where the OVA257 epitope is inserted) mRNA, compared with the PA and presumably PB1 (21). If this is taken as a correlate of protein expression, then there would be less PB1703 and PA224 as a binding site on available K^b MHC class I molecules. The notion that Ag presentation by APCs can influence immunodominance hierarchies has been proposed by numerous studies (2, 7, 8, 20). The limiting nature of Ag presented by APC can result in competition for both Ag/MHC complexes by T cells of the same specificity (8), and/or the available space on the APC for CTL of different specificities (7). Importantly, the constraint on Ag presentation for the APC does not stem from the killing of APCs by specific CTL (8).

In the case of a nonproductive influenza A virus challenge, in which a low influenza A virus dose is used, it would be expected that dendritic cells that are nonproductively infected travel viaafferent lymph (or blood) to the regional lymph nodes and spleen, where they stimulate naive or memory T cell populations (20, 36). Use of the HK-OVA virus results in the recruitment of another, immunodominant T cell response specific for OVA257, leading to competition for available space and/or peptide/MHC complexes on
the APC surface. There were varying levels of immunodominance effects, with the K\(^{p}\)PB1\(_{703}\)- and D\(^{p}\)PA224-specific responses most affected, followed by D\(^{Np}\)NP366-specific response. It is tempting to speculate that this may relate to different levels of presentation of the various peptide/MHC complexes on the surface of APCs. It has been demonstrated that the spectrum of Ag presentation is much more limited for PA224 compared with NP366 (20).

Similarly, when large numbers of naive OT-I K\(^{OVA}\)257-specific T cells were introduced into the responder milieu, a similar hierarchy of immunodominance was observed with the host K\(^{OVA}\)257 most affected, followed by K\(^{p}\)PB1\(_{703}\), D\(^{p}\)PA224, and D\(^{Np}\)NP366. This contrasts with earlier experiments in which transfer of large numbers of Tg T cells resulted in diminished host responses only of the same specificity (7, 8). The suggestion was that responder T cells compete for sites on APCs in an epitope-specific fashion (7, 8). The difference in susceptibility to immunodominance between D\(^{p}\)PA224 and D\(^{Np}\)NP366 may reflect peptide affinity for MHC. This seems unlikely as measurement of pMHC stability as a correlate for peptide affinity showed that D\(^{p}\)PA224 has higher affinity than D\(^{Np}\)NP366 for H2D\(^{b}\) (22) (N. La Gruta, P. C. Doherty, and S. J. Turner, submitted for publication). Therefore, the increased susceptibility of D\(^{p}\)PA224 responses to immunodomination does not reflect peptide affinity for MHC. This supports earlier findings suggesting that peptide affinity plays only a minor role in determining immunodominance hierarchies (2, 22). Perhaps the fact that clonal expansion of the K\(^{p}\)PB1\(_{703}\)-specific T cells also seems to be relatively diminished is fortuitous, and simply reflects that this was the smallest response analyzed. An alternative explanation is that there is some level of competition for binding of both OVA\(_{257}\) and PB1\(_{703}\) to the available K\(^{n}\) molecules within the APC population. Such competition might reflect differences in either peptide affinity for the K\(^{n}\) molecule, differences in efficiency of epitope processing and presentation, or both (2). Combined with the increased OT-I precursor frequency, this results in an almost complete abrogation of the PB1\(_{703}\)-specific response.

Importantly, unlike earlier studies (7), transfer of small numbers of OT-I CTL still significantly diminished the host responses to K\(^{OVA}\)257, K\(^{p}\)PB1\(_{703}\), D\(^{p}\)PA224, and D\(^{Np}\)NP366. This, together with the fact that the responses to the K\(^{p}\)PB1\(_{703}\) and D\(^{p}\)PA224 could not be rescued by coinfection with HK virus, demonstrates that the OT-I CTL are potent inhibitors of subdominant CTL responses. All of the OT-I cells in mice that received low numbers of Tg cells underwent >8 cellular divisions, while this was not the case in mice that had received high numbers of OT-Is (data not shown). Despite this, the overall magnitude of the OT-I responses was similar, suggesting that not all of the OT-Is transferred at high numbers were used. So, transfer of low numbers of OT-Is was still above a threshold where they could still demonstrate potent inhibition of the host response to other influenza A virus epitopes after infection. Importantly, simultaneous infection with both wild-type HK and HK-OVA viruses did not fully overcome the immunodominance of D\(^{p}\)PA224 and K\(^{p}\)PB1\(_{703}\) responses by OT-I CTL. This suggests that in this model, naive T cell precursor frequency is a more significant factor contributing to immunodominance rather than the amount of Ag presented. It was interesting to note that a proportion of transferred OT-I were capable of making IFN-\(\gamma\) upon peptide stimulation despite not receiving Ag-specific stimulation (data not shown). Perhaps a reason for the potency of OT-I competition is they are able to be recruited and acquire effector function very early. It has been suggested that early production of IFN-\(\gamma\) can provide a competitive advantage for a given CTL population, enabling this population to dominate the T cell response to infection (6). Such early competition is likely to impact on those epitopes where Ag will be more limited. Another possible explanation for the immunodominance is better viral control due to early recruitment of OT-I CTL lowering overall levels of Ag. The antiviral effects of IFN-\(\gamma\) production by OT-Is may play a role in explaining why after coinfection with HK and HK-OVA viruses, the D\(^{Np}\)NP366-specific response was restored in the infected lung, yet the D\(^{p}\)PA224-specific response was only partially restored. IFN-\(\gamma\) production by OT-Is could limit replication of both viruses in the lung. As there is more NP than PA produced per virion after infection (21), coinfection with both HK-OVA and HK possibly still results in enough NP366 but not enough PA224 to overcome the suppressive effects of the OT-I CTL.

Overall, the results are in accordance with the idea that altering the size of the precursor pool for a particular T cell set can modify unrelated virus-specific CD8\(^{+}\) T cell immunodominance hierarchies (2, 7, 8, 21). In general, it seems that the factors determining the magnitude of any particular epitope-specific CD8\(^{+}\) T cell response are robust. Although such profiles can be changed by emphasizing or eliminating other Ag-specific CD8\(^{+}\) sets, the magnitude of the effect is likely to be such that the effective development of either T cell memory or a protective recall response will not be substantially compromised.

Acknowledgments
We thank Drs. Nicole La Gruta, Katherine Kedzierska, John Stambas, and Paul Thomas for critical review and discussion, and Dina Stockwell and Yolanda Sims for excellent technical assistance.

Disclosures
The authors have no financial conflict of interest.

References
15. Belz, G. T., W. Xie, J. D. Altman, and P. C. Doherty. 2000. A previously unrecognized H-2D\(^{b}\)-restricted peptide prominent in the primary influenza A virus-


