Immunity, Vol. 8, 683–691, June, 1998, Copyright ©1998 by Cell Press

Virus-Specific CD8$^+$ T Cells in Primary and Secondary Influenza Pneumonia

Kirsten J. Flynn,* Gabrielle T. Belz,*
John D. Altman,† Rafi Ahmed,†
David L. Woodland,* and Peter C. Doherty*‡
*Department of Immunology
St. Jude Children's Research Hospital
Memphis, Tennessee 38105
†Emory Vaccine Center
Department of Microbiology and Immunology
Emory University School of Medicine
Atlanta, Georgia 30322

Summary

Virus-specific CD8$^+$ effector T cells (eCTL) are enriched in the lungs of mice with primary influenza pneumonia, though later detection of memory T cells (mCTL) in the mediastinal lymph nodes (MLN) or spleen by peptide-based staining protocols is at the limits of flow cytometric analysis. Respiratory challenge with an H3N2 virus months after H1N1 priming induces a massive recall response, which reduces virus titers 2–3 days earlier than in nave controls. Influenza-specific mCTL produce interferon-γ within 6 hr, but still take 4–5 days to localize to the infected respiratory tract. The delay reflects that the recall response develops first in the MLN, which contains relatively few mCTL. The response to a subdominant epitope is less obvious after secondary challenge.

Introduction

The outcome of infection in a previously unexposed individual is generally considered to reflect a race between the development of cell-mediated immunity and the rate of virus growth (Doherty et al., 1992). The greater the number of virus-infected cells at the stage when the virus-specific CD8$^+$ cytotoxic T lymphocyte effectors (eCTL) leave the regional lymph nodes and travel via the blood to the site(s) of virus replication in somatic tissues, the less favorable the clinical outcome is likely to be. The techniques for measuring both the extent of virus replication and the numbers of infected cells are both extremely sensitive and long established. Until very recently, the same could not be said for the quantitation of the CD8$^+$ CTL response, the other half of the equation. Though CD8$^+$ T cells recovered from sites of virus-induced pathology show evidence of potent virus-specific CTL activity (Zinkernagel and Doherty, 1973; Allan et al., 1990; Hou et al., 1992), it had not been possible to determine the actual eCTL numbers.

The best method available, limiting dilution analysis (LDA) utilizing a 5–7 day microculture protocol, generally indicated that $<$1:50 of the extravasated CD8$^+$ T cells are specific for the inducing pathogen (Doherty et al., 1996). Such values had long been thought to underestimate the size of the eCTL population. The assumption was that LDA may provide a reasonable measure of the CD8$^+$ memory T cells (mCTL) that can be expanded through 10 or more rounds of in vitro culture, while further stimulation of the virus-specific eCTLs probably leads to activation-induced cell death (Iezzi et al., 1998). For example, in mice primed several months previously with a parainfluenza type 1 virus (Sendai virus) and then challenged intranasally (i.n.) with an influenza A virus, the influenza-specific CTL precursor (pCTL) frequencies in the inflammatory cells recovered by bronchoalveolar lavage (BAL) were only 2- to 3-fold higher than those for the Sendai-specific memory set (Tripp et al., 1995b). However, such BAL populations contain potent eCTLs specific for influenza virus but not for Sendai virus.

The development of new techniques for staining antigen-specific CD8$^+$ T cells with tetrameric complexes of MHC class I glycoprotein+peptide, or stimulating T cells with peptide and then analyzing for the synthesis of interferon-γ (IFNγ), has greatly facilitated the dissection of cell-mediated immunity (Altman et al., 1996; Busch et al., 1998; Butz and Bevan, 1998; Murali-Krishna et al., 1998). Experiments using one or more of these approaches to probe human peripheral blood lymphocytes from individuals infected with human immunodeficiency virus (HIV) and spleen cells from mice given lymphocytic choriomeningitis virus (LCMV), have shown very clearly that LDA underestimates the prevalence of virus-immune CD8$^+$ T cells in both acute and persistent infections by a factor of at least 10-fold (Altman et al., 1996; Butz and Bevan, 1998; Murali-Krishna et al., 1998).

The present analysis describes the dual application of the tetramer-staining and peptide stimulation approaches to quantitate the primary and secondary CD8$^+$ CTL response in mice with influenza pneumonia, being a description of the events occurring in a nonlymphoid site of virus-induced pathology. The results establish that the influenza-specific CD8$^+$ eCTL numbers are much greater than previously suspected (Tripp et al., 1995b; Doherty et al., 1996) and illustrate very clearly the durability, benefits, and limitations of virus-specific CD8$^+$ T cell memory.

Results

Specificity of the Tetramer-Staining Reaction

Tetrameric complexes of H-2D^b + Sendai virus NP$_{324–332}$ (SEV9) or H-2D^b + influenza A virus NP$_{366–374}$ (NPP) were used to stain virus-specific hybridoma T cell lines (Deckhut et al., 1993; Cole et al., 1997) of the corresponding specificity (Figure 1A). The reactions in both cases were specific for the appropriate T cells, showing no significant overlap in the extent of binding. The NPP tetramer was then used to probe the inflammatory lymphocytes obtained (Allan et al., 1990) by BAL of mice challenged i.n. 8 days previously with either the HKx31 (H3N2) influenza A virus or with the B/HK influenza B virus that does not contain the NP$_{366–374}$ epitope (Allan et al., 1993). While

‡To whom correspondence should be addressed (e-mail: peter.doherty@stjude.org).

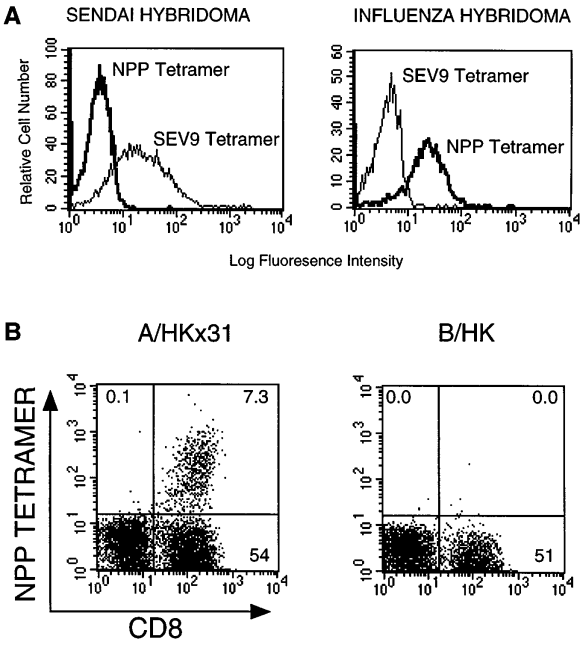

Figure 1. Specificity of the Staining Reaction with the NPP Tetramer

(A) The influenza H-2D^b+NP$_{366–374}$-specific and Sendai H-2D^b+NP$_{324–332}$-specific T cell hybridomas were assayed by flow cytometry for staining with either the NPP (bold line) or SEV9 (thin line) tetramers.

(B) The inflammatory BAL population was obtained from B6 mice 8 days after i.n. infection with either HKx31 or B/HK influenza A or B virus, stained for surface CD8 and the NPP tetramer, and examined by flow cytometry. The numbers given show the percentage of BAL cells in the lymphocyte/lymphoblast gate falling within the respective quadrants. The proportion of CD8$^+$ T cells staining with the NPP tetramer on day 8 was consistent in five independent experiments.

the percentage of CD8$^+$ T cells in the total lymphocyte pool obtained by BAL was comparable between the two infections, approximately 1:8 of the CD8$^+$ T cells in the BAL of the HKx31-primed mice stained with the NPP tetramer. The value for the mice given the B/HK virus was <0.1% (Figure 1B). Other experiments (data not shown) established that the NPP tetramer does not bind to CD4$^+$ T cells in the BAL of mice infected with the HKx31 virus. Thus, staining with the NPP tetramer is very specific, both for a T cell hybridoma maintained in vitro and for highly activated inflammatory cells obtained directly from the pneumonic lung.

Quantitation of Virus-Specific CD8$^+$ T Cells in the Pneumonic Lung

Preliminary studies (Figure 1B and unpublished data) indicated that the prevalence of NPP$^+$CD8$^+$ T cells in the BAL was >10-fold higher than suggested from previous determinations of CTLp frequency. The next experiment (Figure 2) compared the kinetics of CD8$^+$ T cell localization and retention to the BAL following i.n. HKx31 (H3N2) challenge of immunologically naive mice (Figure 2A, primary) and mice that had been primed 8 months previously by intraperitoneal (i.p.) injection of the A/PR/8/34 (PR8, H1N1) virus (Figure 2B, secondary). The HKx31 virus is a recombinant that expresses the surface hemagglutinin (H) and neuraminidase (N) proteins of A/Aichi (H3N2) and the internal components of PR8 (Kilbourne, 1969). The neutralizing antibody response to the surface H and N glycoproteins of these two viruses is not cross-reactive, so the magnitude of the secondary antigenic challenge is, prior to the development of eCTL activity, in every sense equivalent to that occurring in the primary response. The top figures in the upper right quadrant of the flow cytometry profiles shown in Figure 2 give the percentage of the BAL population that stains for both CD8 and NPP, those below estimate the numbers (per mouse) of NPP$^+$CD8$^+$ cells in the BAL, while the bottom value is the SEV9 tetramer-staining control.

Significant numbers of NPP$^+$CD8$^+$ T cells were first detected in the BAL on day 7 in the primary response and on day 5 after secondary challenge, confirming earlier impressions from eCTL assays that established T cell memory confers (at best) a 2–3 day advantage in the rate of effector T cell development and localization to the influenza virus–infected respiratory tract (Bennink et al., 1978). The secondary response has long been known to be greater in overall magnitude. What could not, however, be predicted from any previous analysis was that >15% of the BAL CD8$^+$ T cells in the primary response and >65% in the secondary response were specific for the immunodominant H-2D^b+NP$_{366–374}$ (Townsend et al., 1986). The numbers of inflammatory CD8$^+$ NPP$^+$ T cells present at the peak of the inflammatory process on day 10 were 5-fold higher in the secondary response (2×10^5 cf. 1×10^6).

Comparable profiles for the localization and accumulation of NP-specific CD8$^+$ T cells to the virus-infected respiratory tract were recorded from the same experiment (as in Figure 2) analyzed using the peptide/Brefeldin A/IFNγ protocol (Figure 3). Again, the massive secondary response for CD8$^+$IFNγ^+ lymphocytes is apparent 2 days earlier, and the overall magnitude is about 5-fold greater than for the primary. More than 60% of the inflammatory CD8$^+$ T cells in the challenged mice are IFNγ-producing H-2D^b+NP$_{366–374}$-specific eCTL or the precursors of such eCTL.

Further sets of experiments confirmed the observations for staining BAL cells with the NPP tetramer and for stimulation with the NP$_{366–374}$ peptide in mice challenged with the HKx31 virus and also determined the prevalence of CD8$^+$IFNγ^+ T cells specific for the minor H-2K^b-NS2$_{114–121}$ epitope (Vitiello et al., 1996) in primary and secondary viral pneumonia (Table 1, BAL columns). The mice utilized in Table 1 had been immunized with the PR8 virus 56 days (Expt. 1), 42 days (Expt. 2), or 27 days (Expt. 3; Table 1) previously, compared with 240 days for the group analyzed in Figures 2 and 3. Taking the results together indicates that the recall response may be slightly delayed (though still very substantial), with increasing time following the initial exposure to virus (Figure 2 and Table 1) as influenza H-2D^b + NP$_{366–374}$-specific CD8$^+$ T cells were detected in the BAL as early as 3 days after challenge in the younger mice (Table 1, 45 days). Earlier studies indicate that the primary eCTL response also develops more slowly in older mice, though there is no obvious age-related difference in growth characteristics for the influenza A viruses (Bender et al., 1995).

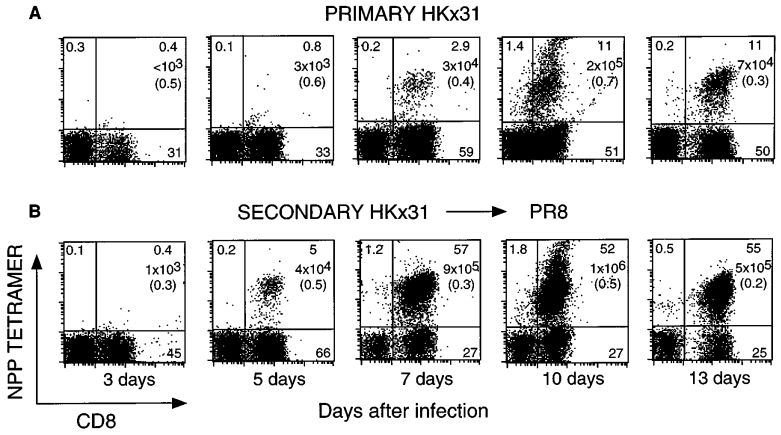

Figure 2. Visualization of CD8+NPP+ T Cells in BAL Population Recovered from Primary or Secondary Influenza Pneumonia

Naive (A) or PR8-immune (B) mice (primed for 8 months) were infected i.n. with the serologically distinct HKx31 virus. The BAL populations collected from each group of mice (n = 9–10) were pooled and examined by flow cytometry for surface staining with anti-CD8α and the NPP tetramer. The percentage of BAL cells in the lymphocyte/lymphoblast gate is shown for each of the quadrants. The other numbers in the upper right-hand gate are an estimate of the average/mouse counts for the $CD8^+$ NPP^+ set and (in brackets) the percentage of live BAL cells staining with the SEV9 tetramer.

Response Profiles for the Minor Epitope

The numbers of T cells specific for the subdominant H-$2K^b$+$NS2_{114–121}$ epitope were relatively higher in the primary than the secondary response (Table 1, NS2-IFNγ and BAL). Comparing the results following stimulation with the two peptides indicated that the eCTL counts for this epitope were at least 33% of those for the dominant H-$2D^b$+$NP_{366–374}$ response on days 7 and 13 in the primary compared with <6% in the secondary (Table 1). The greater prominence of minor NS2 epitope in the primary response was confirmed in further experiments with individual mice (Table 2, B6 mice). The numbers of NP-specific eCTL were significantly increased following secondary challenge, while this was not the case for the NS2-specific population that remained at a comparable, or lower, frequency (Table 2).

The HKx31→PR8 challenge experiment (Table 1; Table 2, B6 mice) left open the possibility that some difference between these two viruses might influence the immunodominance hierarchy. We thus repeated the analysis using an HKx31 challenge in HKx31-primed $Ig^{-/-}$ μMT mice (Kitamura et al., 1991; Topham and Doherty, 1998), to avoid any possible confounding effect due to neutralizing antibody. Again, the prevalence of the NP-specific eCTL in the BAL increased dramatically in the secondary response, while there was no change in frequency for the NS2-specific set (Table 2, μMT mice). The same effect was seen for the mediastinal lymph nodes (MLN) (see Footnote to Table 2). Thus, though the H-$2K^b$+$NS2_{114–121}$-specific T cells were detectable in the BAL as early as 5 days after secondary challenge, they did not show the dramatic expansion in numbers seen for the H-$2D^b$ + $NP_{366–374}$ set.

Virus-Specific $CD8^+$ T Cells in Lymphoid Tissue

Though as many as 10% of the $CD8^+$ mCTL in lymphoid tissue may be specific for LCMV in the very long term (Butz and Bevan, 1998; Murali-Krishna et al., 1998), the influenza-$NP_{366–374}$-specific mCTL in MLN and spleen were at the limits of detection for both peptide-based flow cytometry assays within 6 weeks of i.p. priming (Table 1, days 56 and 42 and MLN and spleen). The $CD8^+$ set was enriched prior to counting to facilitate the FACS analysis by decreasing the number of events acquired. The frequencies of H-$2D^b$+$NP_{366–374}$-specific mCTL for resting mice ranged from 1:600 to 1:1400 when corrected to total spleen or MLN lymphocyte counts, the convention used previously for the LDA studies with the influenza model (Doherty et al., 1996). This is generally higher than previous findings for LDA with whole

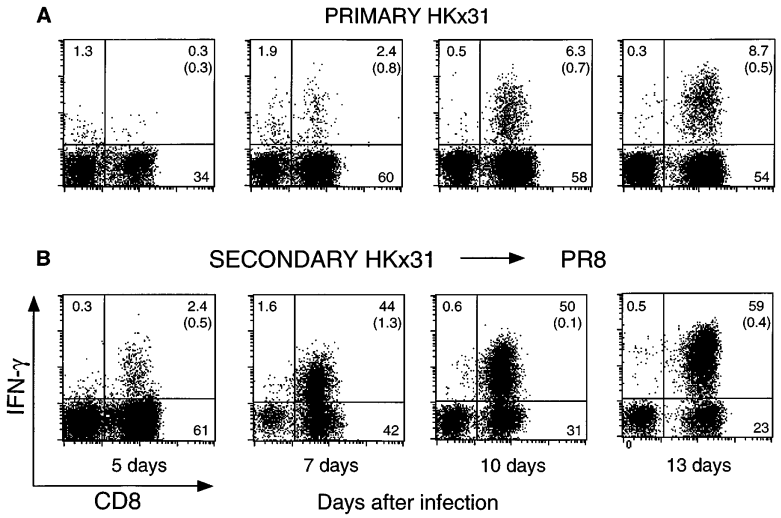

Figure 3. Quantitation of the Primary and Secondary Viral Pneumonia by Short-Term Stimulation with Peptide and Staining for IFNγ

This analysis utilized the same mice that were assayed in Figure 2. The BAL cells were incubated for 6 hr in Brefeldin A, the $NP_{366–374}$ peptide, stained for CD8α, and then permeabilized by fixation and stained for IFNγ. The percentage of BAL cells in the lymphocyte/lymphoblast gate is shown for the respective quadrants, together with (brackets) the percentage of BAL cells staining for IFNγ after incubation in the absence of $NP_{366–374}$.

Table 1. Comparison of Primary and Secondary Influenza-Specific $CD8^+$ T Cell Responses in the BAL, MLN, and Spleen

Days After Infection[a]		$CD8^+$ T Cells Staining (%)								
PR8	HKx31	NPP[b]			NP-IFNγ			NS2-IFNγ[b]		
i.p.	i.n.	BAL	MLN	Spleen	BAL	MLN	Spleen	BAL	MLN	Spleen
Experiment 1										
—	7	4.7	0.3	0.5	6.7	0.1	0.5	2.8	0.2	0.1
—	13	10	1.5	1.2	6.9	0.4	0.4	5.1	0.2	0.2
56	—	NT	0.3	0.9	NT	0.2	0.6	NT	<0.1	0.1
63	7	81	7.0	24	65	6.8	16	0.8	0.2	0.6
69	13	80	17	39	77	14	21	0.7	0.6	0.3
Experiment 2										
—	3	<0.1	<0.1	<0.1	1.5	0.2	0.1	<0.1	0.5	0.1
—	5	0.4	<0.1	<0.1	0.9	0.4	0.1	1.3	0.7	0.2
—	7	3.4	0.1	0.2	6.7	0.4	0.3	2.2	0.2	0.1
42	—	NT	0.2	0.8	NT	0.2	0.8	NT	<0.1	0.2
45	3	2.7	<0.1	0.2	3.9	0.4	0.4	0.7	0.1	0.1
47	5	40	8.5	1.3	33	7.2	1.4	3.4	0.7	0.6
49	7	67	9.9	26	58	12	23	3.7	1.4	1.4
Experiment 3										
—	5	NT	0.2 ± 0.1	0.1	NT	0.6 ± 0.3	0.1 ± 0.1	NT	0.3 ± 0.2	0.1 ± 0.1
27	—	NT	0.6	2.0	NT	0.5	1.5	NT	0.2	0.3
32	5	NT	9.5 ± 5.2[c]	1.4 ± 0.5	NT	9.3 ± 4.3[c]	1.3 ± 0.4[c]	NT	0.5 ± 0.2[c]	0.2 ± 0.1

[a] Naive B6 mice or mice primed i.p. with $10^{7.9}$ EID_{50} of the PR8 (H1N1) influenza A virus were challenged i.n. 56, 42, or 27 days later with $10^{6.8}$ EID_{50} of the serologically different HKx31 (H3N2) virus.

[b] The $CD8^+$ T cells were stained directly with the NPP tetramer, or stimulated with the $NP_{366–372}$ or $NS2_{114–121}$ peptides for 6 hr in the presence of Brefeldin A, fixed, and stained for IFNγ. The BAL cells were first adhered on plastic to remove macrophages, while the MLN and spleen cells were enriched for the $CD8^+$ set to >75% purity by removing the $CD4^+$ T cells, B cells, and MHC class II^+ cells using Dynabeads plus the TIB120 and GK1.5 MAbs.

NT, not tested.

[c] Significantly greater ($p < 0.001$) than the values for the spleen. The data showing SD values is for five individual mice. The assays were done as described in the Experimental Procedures.

virus (generally about 1:3000), but the difference is not as extraordinary as that found for LCMV (Butz and Bevan, 1998; Murali-Krishna et al., 1998).

The peak frequencies detected at any stage (Table 1, NS2-IFNγ and MLN and spleen) for the minority $CD8^+IFN\gamma^+$ H-$2K^b$+$NS2_{114–121}$-specific set following i.n. challenge with the HKx31 virus were 0.7% of (primary) and 1.4% (secondary). Maximal counts for the immunodominant $NP_{366–374}$-specific $CD8^+$ T cells were recorded in the MLN and spleen on day 13 of the primary (1.5% and 1.2%) and secondary (17% and 39%) responses, respectively (Table 1, day 13 and day 69 and NPP^+ and NP-$IFN\gamma^+$). The comparable values for day 7 after the HKx31 challenge (Table 1, days 7, 63, and 49) were 0.3 and 0.5% (primary) and 9.9% and 26% (secondary). The most surprising finding from these results is that the prevalence of splenic $CD8^+$ T cells specific for the immunodominant H-$2D^b$+$NP_{366–374}$ epitope is very high (Table 1, day 69) long after the time that infectious virus would normally have been eliminated from these mice (Allan

Table 2. Immunodominance Hierarchies in the BAL

			$CD8^+$ T Cells Staining (%)	
Mouse Strain	Day	Priming and Challenge[a]	NP-IFNγ	NS2-IFNγ
B6	9	HKx31	20.8 ± 9.9	5.8 ± 1.8[c]
		HKx31 → PR8	69.4 ± 19.5[b]	3.7 ± 4.1[c]
	12	HKx31	19.2 ± 10.8	4.7 ± 1.2[c]
		HKx31 → PR8	79.1 ± 1.5[b]	1.7 ± 1.2[b,c]
μMt[d]	10	HKx31	24, 16	11, 4
		HKx31 → HKx31	76, 82	10, 4

[a] The B6 mice were primed i.p. with the PR8 virus 1 month prior to challenge, while the μMT mice had been given the standard i.n. dose of HKx31 8 months previously. Naive controls were challenged concurrently with the HKx31 virus. Groups of four or five mice were used in a contemporary comparison with the B6 mice. The results for the μMT mice are for two naive and two primed individuals. The experimental protocol and the peptide/BrefeldinA/IFNγ assay system is described in the Experimental Procedures and in the legend to Table 1.

[b] Significantly different ($p < 0.01$) from the value for the primary response.

[c] Significantly different ($p < 0.01$) from the value for the immunodominant NP epitope.

[d] The response profiles for the MLN were also measured for the μMT mice. The values were NP: primary, 1.6, 1.9; secondary, 12.4, 15.4; NS2: primary, 1.4, 1.5; secondary, 2.3, 1.5.

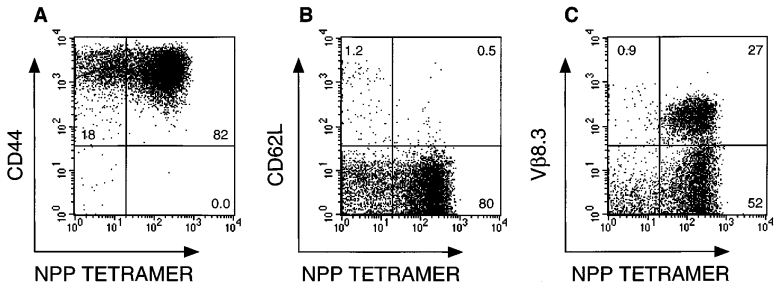

Figure 4. Activation Phenotype of the CD8+ T Cells in the BAL

The BAL population was obtained from secondarily challenged mice on day 13 and assayed by flow cytometry for surface staining with anti-CD8, NPP tetramer, and (A) CD44, (B) CD62L, or (C) Vβ8.3. The percentage of BAL cells in the lymphocyte/lymphoblast $CD8^+$ gate is shown for each of the quadrants.

et al., 1990). This effect was not apparent in prior LDA studies (Tripp et al., 1995b) and the H3N2 influenza A viruses cannot generally be isolated from mouse spleen at any stage of the disease process (Eichelberger et al., 1991).

However, despite the fact that the H-$2D^b$+$NP_{366–374}$-specific $CD8^+$ T cells were present at highest prevalence in the spleen from day 7 after the HKx31 challenge (Table 1, days 63 and 49) the antigen-driven increase in virus-specific $CD8^+$ T cell numbers was initially more prominent in the MLN (Table 1, day 47). This skewing of the host response to the MLN on day 5 was confirmed in Expt. 3, where five individual mice were compared simultaneously (Table 1, day 32). The findings support the idea that, although more mCTL are present in the resting spleen than in the MLN (Table 1, days 56, 42, and 27), the drainage of antigen-presenting dendritic cells via afferent lymph triggers the secondary eCTL response in the regional lymph node rather than the spleen (Table 1, day 32). This compartmentalization effect is also apparent for the primary response (Table 1, day 5, Expt. 3, NP-IFNγ), and for the secondary response to the minority H-$2K^b$+$NS2_{114–121}$ epitope (Table 1, day 32 and NS2-IFNγ).

Phenotype of the Virus-Specific T Cells

The BAL $CD8^+NPP^+$ population showed the $CD44^{Hi}$ $CD62L^{Lo}$ profile characteristic (Hou and Doherty, 1993; Tripp et al., 1995a) of activated T cells (Figures 4A and 4B) from the time of initial localization to the virus-infected lung (Figure 2) on day 5 (secondary) or day 7 (primary). Earlier cell-sorting experiments established that this phenotype, which was maintained throughout the response (data not shown), is characteristic of both the influenza virus–specific eCTL killer population and the mCTL set that can be expanded by LDA (Tripp et al., 1995a). In fact, all of the lymphocytes (including the $CD8^+$ T cells, Figure 4A) recovered by BAL were consistently $CD44^{Hi}$. This may reflect that CD44 expression is required to mediate lymphocyte extravasation into sites of virus-induced inflammatory pathology (DeGrendele et al., 1997). Different profiles were found for the MLN and spleen, where approximately 20% (primary) or 50% (secondary) of total $CD8^+$ T cells were $CD44^{Hi}$ on day 7 after infection (data not shown). Similarly, the staining profiles for the lymph node homing receptor (CD62L) partitioned the $CD8^+$ T cells in lymphoid tissue into $CD62L^{Lo}$ and $CD62L^{Hi}$ subsets. Both populations were also found in the BAL, though the $CD62L^{Lo}$ set was much more prevalent (Figure 4B). However, at all times examined and in all tissues, the very great majority of the $CD8^+NPP^+$ T cells were $CD44^{Hi}CD62L^{Lo}$. This continued to be true for spleen cells from B6 mice primed 2 months previously with the PR8 virus (data not shown), confirming patterns determined previously by LDA.

Previous flow cytometric analysis, LDA subsequent to FACS enrichment, and the generation of a range of hybridoma T cell lines all indicated that the Vβ8.3 T cell receptor (TCR) chain is utilized at high frequency (Deckhut et al., 1993) in the response to H-$2D^b$+$NP_{366–374}$. Sequencing studies with the hybridomas indicated that this response is polyclonal within the $V\beta8.3^+$ set (Deckhut et al., 1993). Direct staining for a spectrum of 16 different TCRVβ and 3 TCRVα chains confirmed that Vβ8.3 (Figure 4C) was selectively overexpressed on the $CD8^+NPP^+$ (cf. $CD8^+NPP^-$) BAL population (data not shown). The only other TCR chain detected at enhanced frequency on the $CD8^+NPP^+$ lymphocytes recovered from mice with primary or secondary influenza pneumonia was Vβ4 (data not shown).

The Extent of Protection

The results overall (Figures 2 and 3 and Tables 1 and 2) illustrate very graphically the durability of established $CD8^+$ T cell memory to the influenza A viruses and confirm earlier impressions of the limitations of the $CD8^+$ T cell recall response as a sole protective mechanism (Andrew et al., 1987; Doherty et al., 1989; Lawson et al., 1994). The virus has at least 2–3 days to become established in the respiratory tract before the consequences of prior exposure are apparent (Figure 2 and Table 1). Challenging HKx31 immune mice with the more virulent PR8 virus led to a >100-fold reduction in lung virus titers on day 6 when compared with primary PR8 infection (Table 3). However, though the immune mice showed clear evidence of a secondary $CD8^+$ T cell response, in that greater numbers of $CD8^+NPP^+$ T cells were detected earlier in the BAL, all of the HKx31 immune and naive animals still succumbed to the PR8 infection. Influenza-specific $CD8^+$ T cells function in vivo via a perforin/granzyme or Fas-mediated cytotoxic mechanism (Topham et al., 1997), so even though the virus-infected cells may be eliminated, the degree of CTL-mediated damage to the respiratory epithelium may not be survivable if the virus has disseminated too extensively prior to the recall to eCTL function.

A difference between the PR8→HKx31 (Table 3) and the HKx31→PR8 (Figures 2 and 3 and Table 1) challenge experiments was that the secondary response induced in the BAL by the lower dose of the more virulent PR8 virus looked to be smaller in magnitude than that caused

Table 3. Early Control of Virus in the Secondary Response

	% NPP$^+$ CD8$^+$ T Cells in BAL		Virus titer in Lung (log_{10} EID_{50})
PR8[a] Challenge	Day 5	Day 6	Day 6
Primary	0.1 ± 0.01	0.5 ± 0.05	>6.5 ± 0.0
Secondary	3.5 ± 0.2	14.4 ± 0.2	4.3 ± 0.6

[a] The mice were primed i.n. with the HKx31 virus and then, together with naive controls (primary), challenged i.n. 2 months later with $10^{4.0}$ EID_{50} of PR8 (secondary). Groups of five mice were assayed at each timepoint.

by HKx31. A further experiment with the HKx31→PR8 model gave log_{10} EID_{50} lung titers of 5.7 ± 0.1 and 5.9 ± 0.6 on day 3 and 6.0 ± 0.4 and 3.9 ± 0.3 on day 5 for the primary and secondary responses, respectively. Again, the protective effect (Table 3) of established CD8$^+$ T cell memory was delayed in onset, though it did lead to a substantial (100-fold) early reduction of virus titers in the lung. Other studies with Ig$^{-/-}$ μMT mice have also shown that the secondary CD8$^+$ T cell response to the relatively mild HKx31 virus is highly protective (Topham and Doherty, 1998).

Discussion

Analysis of the BAL population with both the influenza NPP tetramer and by staining $NP_{366–374}$-specific T cells for IFNγ has provided rigorous, quantitative evidence that very substantial numbers of virus-specific CD8$^+$ T cells are present in a nonlymphoid site of virus-induced inflammatory process. Application of the same technology established previously that the prevalence of virus-specific CD8$^+$ T cells in the spleen during the course of primary LCMV infection is extremely high (Murali-Krishna et al., 1998). At the peak of the response, more than 50% of the splenic CD8$^+$ T cells are specific for the two prominent LCMV epitopes recognized in H-2^b mice. By contrast, influenza-specific CD8$^+$ T cells are found in much lower numbers in the regional lymph nodes and spleen (1%–2%), though the BAL CD8$^+$ T cells show frequencies (20%–70%) comparable to those found for the LCMV-specific set in lymphoid tissue. This presumably reflects the differential distribution of antigen in these virus infections.

While LCMV replicates extensively in the lymph nodes and spleen (Buchmeier et al., 1980; Ahmed et al., 1987), productive infection with the influenza A viruses is substantially restricted to the lung (Eichelberger et al., 1991; Hamilton-Easton and Eichelberger, 1995). The generation of infectious influenza A viruses depends on the activity of a trypsin-like enzyme that functions to cleave the precursor of the viral hemagglutin molecule (Walker et al., 1992; Horimoto and Kawaoka, 1995). In mammals, the distribution of this enzyme seems to be largely restricted to the respiratory epithelium. Much of the stimulus in the regional lymph nodes and spleen may result from the migration of dendritic cells (Hamilton-Easton and Eichelberger, 1995) that are infected in the respiratory tract (Holt et al., 1993; McWilliam et al., 1997) and support a full virus life cycle, with the exception that no infectious progeny are produced. This means that, as there should be no secondary spread in lymphoid tissue, the antigen load in this site will be much lower for influenza than for LCMV. The consequence is that potent eCTL tend to be found only in the BAL of mice following primary i.n. infection with the HKx31 virus (Allan et al., 1990) but are readily detected in the lymph nodes and spleen of those given LCMV (Zinkernagel and Doherty, 1974; Lynch et al., 1989).

The clonal expansion of the virus-specific CD8$^+$ set and the prevalence of mCTL that can be stained with the tetrameric complexes is also much higher following primary infection with LCMV than with influenza. The influenza-specific NPP$^+$CD8$^+$ set is maintained at a level of approximately 1:125 of the CD8$^+$ population in the spleen and 1:300 in the MLN, after the acute phase of the host response is terminated. The comparable calculation for LCMV-specific CD8$^+$ mCTLs is closer to 1:10. Again, the difference could be thought to reflect the extent of the clonal burst during the antigen-driven phase of the host response (Hou et al., 1994). The caveat to the interpretation that differential antigen concentration is the principal determinant of clonal burst size is that the frequencies demonstrated by tetramer staining of enriched CD8$^+$ T cells from spleens of mice infected with an intracellular bacterium (*Listeria monocytogenes*) that replicates in the lymphoid compartment are more comparable to those found for influenza than for LCMV (Busch et al., 1998). A further possibility is that at least some of the difference in magnitude for the responses to LCMV (a natural mouse pathogen) and influenza virus, which does not normally infect mice, may reflect the extent of the naive T cell repertoire and thus the magnitude of the pCTL pool (Busch et al., 1998).

It is possible that, following the establishment of a substantial mCTL population, the prevalence of antigen-presenting cells is a less critical determinant of the magnitude of the secondary influenza-specific response in the spleen. The HKx31 influenza A virus used for the majority of the secondary challenge experiments causes little, if any, productive infection in lymphoid tissue (Hamilton-Easton and Eichelberger, 1995) and is generally eliminated from the lung by day 7 in Ig$^{-/-}$ μMT mice with a primed CD8$^+$ T cell compartment (Topham and Doherty, 1998). Perhaps, as suggested from earlier LDA experiments (Tripp et al., 1995c), the continued proliferation of highly activated T cells is (for a time) independent of antigen. However, little is known about the persistence of antigen-presenting cells in this system (Hamilton-Easton and Eichelberger, 1995). An alternative explanation for the massive numbers of CD8$^+$NPP$^+$ lymphocytes in the spleens of the secondarily challenged mice could be that many of these are inflammatory T cells that have exited the lung. Clearly, these insights into the magnitude of the influenza virus–specific CD8$^+$ T cell response raise many questions that require further, detailed analysis.

The current experiments with the influenza A viruses leave no doubts concerning the durability and extent of virus-specific $CD8^+$ T cell memory (Hou et al., 1994; Lau et al., 1994; Mullbacher, 1994; Ahmed and Gray, 1996; Doherty et al., 1996). Mice primed 8 months previously with the PR8 (H1N1) virus generate a very potent secondary $CD8^+$ T cell response following i.n. challenge with the serologically distant HKx31 (H3N2) virus that shares the $NP_{366–374}$ epitope (Figure 2). There is no evidence that influenza A virus persists in any sense in mammalian hosts, though the possibility that (as reported recently for LCMV) some viral genetic elements may be copied back into the genome by retroviral reverse transcriptase (Klenerman et al., 1997) is yet to be investigated. This seems unlikely, as productive or nonproductive infection with the influenza A viruses inevitably leads to cell death.

It is intriguing that mCTL from the lymph nodes and spleen can be induced to secrete substantial amounts of IFNγ within 6 hr of in vitro peptide restimulation (Table 1), as shown previously for influenza-specific mCTL from human peripheral blood (Lalvani et al., 1997), but take 3 days or more to localize in substantial numbers to the virus-infected respiratory tract. The delay in the recall of eCTL to the lung seems to be a function of the pathobiology of the host response. The fact that the secondary response appears to be initiated in the regional lymph nodes raises the possibility that the mCTL must first be mobilized from the recirculating pool or be recruited to the MLN from the much larger numbers found in resting spleen. The spleen is not subject to the gating mechanism that facilitates the entry of naive $CD62L^{Hi}$ T cells into the lymph nodes via the high endothelial venules (Butcher and Picker, 1996) and concurrently excludes the $CD8^+CD62L^{Lo}$ populations. These mCTLs are generally thought to gain access to the lymph nodes via the afferent lymph, after trafficking through somatic tissues (Mackay et al., 1992).

Enlargement of the regional lymph nodes is an early event in these respiratory virus infections (Doherty et al., 1992). Some of the massive, nonselective lymphocyte recruitment (Allan et al., 1990) that occurs may reflect the direct influence of cytokines, such as the type 1 interferons (Gresser et al., 1981; Korngold et al., 1983; Tough et al., 1996). Perhaps these cytokines constitute (or act to trigger) a damage signal (Matzinger, 1994) inducing the expression of endothelial receptors (Butcher and Picker, 1996) that facilitate the direct extravasation of any recirculating T cells (including the $CD62L^{Lo}$ set) into the lymph node. It is known that CD44-hyaluronate interactions are important for the migration of superantigen-stimulated T cells to sites of inflammatory pathology (DeGrendele et al., 1997). Previous LDA analysis (Tripp et al., 1995a) indicates that resting influenza-specific mCTLs are constitutively $CD44^{Hi}$. The other possibility is that the initiation of the whole secondary response in this localized virus infection is dependent on the relatively small population of resting mCTL detected in the regional lymph nodes. If this is the case, the kinetic factors determining the recall rate for eCTLs to the lung will be determined by the mCTL numbers in the MLN and the time taken for the antigen-presenting dendritic cells to exit the virus-infected respiratory tract via afferent lymph (McWilliam et al., 1997).

In general, the kinetics of the primary response determined by both staining for NPP and induction of IFNγ production with peptide reflect the results of previous eCTL and LDA experiments (Doherty et al., 1978; Allouche et al., 1982; Askonas et al., 1982; Doherty et al., 1996). The same is true for our earlier analyses of TCR and activation phenotypes (Deckhut et al., 1993; Tripp et al., 1995a). The availability of the NPP staining protocol clearly makes the analysis of cell-surface phenotypes for the virus-specific $CD8^+$, which previously had to be approached by LDA subsequent to FACS separation, much more accessible. The major differences are that the prevalence rates for mCTL in lymphoid tissue may be 2- to 5-fold higher with the tetrameric-staining and peptide stimulation approaches than indicated by LDA, we had no previous measure of eCTL numbers, and the antigen-specific component of the secondary response is much more massive than anyone could have predicted. Earlier ideas about the size of both the eCTL and mCTL sets (Doherty et al., 1996) need to be revised, and there is now a very clear picture of how greatly skewed the eCTL/mCTL ratio is during the course of primary and secondary virus-specific T cell responses.

Being able to stain the eCTL population directly also showed that an immunodominance hierarchy characteristic of the primary response may be even more prominent in the inflammatory process following secondary i.n. challenge. A comparable difference between the naive and memory T cell responses was not seen for somewhat similar studies with the *L. monocytogenes* model (Busch et al., 1998). The *Listeria* experiments used an homologous challenge system that could be complicated by the effects of specific antibody to bacterial surface components, and compared T cells at relatively low frequency in the spleen rather than the site of pathology. As predicted by the originators of the approach (Altman et al., 1996; McHeyzer-Williams et al., 1996), many new experimental possibilities, questions, and debates are being opened by the availability of the tetramer technology.

Experimental Procedures

Virus Infection and Mice

The C57BL/6J (B6) female mice were anesthetized with avertin (2,2,2-tribromoethanol) and then infected i.n. with varying egg infectious doses (log_{10} EID_{50}) of different influenza viruses (Allan et al., 1990, 1993). Most experiments were done using a nonlethal challenge ($10^{6.8}$ EID_{50}) with the A/HKx31 (HKx31, H3N2) influenza A virus, a regime that was also used to prime for long-term memory. Mice given 10^4 EID_{50} of the A/PR8/34 (PR8, H1N1) influenza A virus i.n. generally succumbed to the infection, while the majority survived respiratory challenge with 10-fold less virus. Administering $10^{7.9}$ EID_{50} of PR8 i.p. caused no obvious symptoms and was also used to establish memory. Some controls were given a $10^{5.6}$ i.n. dose of the noncross-reactive B/Hong Kong/73 (B/HK) influenza B virus. All immune mice were held for at least 1 month prior to secondary challenge.

Tissue Sampling and Treatment

Inflammatory cells were obtained from anesthetized infected mice by BAL. The BAL cells were first absorbed on plastic petri dishes (Falcon, Lincoln Park, NJ) for 60 min at 37°C to remove macrophages (Allan et al., 1990). In some experiments, the lungs were frozen and later homogenized for virus isolation in embryonated hen's eggs (Allan et al., 1990). Single cell suspensions were prepared from

spleen and MLN, and erythrocytes were lysed. The MLN and spleen populations were enriched for $CD8^+$ T cells (Hou et al., 1994) by treatment with MAbs to CD4 (GK1.5) and MHC class II (TIB120), followed by sheep anti-mouse and sheep anti-rat Dynabeads (Dynal A. S, Oslo, Norway). The final preparations from the MLN and spleen contained 85%–95% and 75%–85% $CD8^+$ T cells, respectively.

Peptides

The H-$2D^b$-binding influenza A peptide $NP_{366–374}$ (Townsend et al., 1986), the Sendai virus peptide $NP_{324–332}$ (Kast et al., 1991), and the H-$2K^b$-binding influenza A peptide $NS2_{114–121}$ (RTFSFQLI) (Vitiello et al., 1996) were synthesized at the Center for Biotechnology, St. Jude Children's Research Hospital (SJCRH), using a Perkin Elmer Applied Biosystems (Berkeley, CA) 433A peptide synthesizer.

The NPP and SEV9 Tetramers

The preparation of tetrameric complexes of MHC class I glycoprotein with viral peptide has been described previously (Murali-Krishna et al., 1998). The experiments utilized the H-$2D^b$ MHC class I glycoprotein complexed with the influenza virus nucleoprotein (NP) ASNENMETM peptide ($NP_{366–374}$) and the Sendai virus $NP_{324–332}$ FAP GNYPAL peptide, being designated NPP and SEV9, respectively. H-$2D^b$-$NP_{366–374}$ is immunodominant in the $CD8^+$ T cell response to the influenza A viruses in H-2^b mice (Doherty et al., 1978; Townsend et al., 1986), while H-$2D^b$- $NP_{324–332}$ is a minor epitope in the response to Sendai virus (Cole et al., 1997). The tetramers were assayed with the Vβ8.3$^+$$CD8^+$ 12.91 influenza A-specific T cell hybridoma (H-$2D^b$+NP365–380) derived from B6 mice (Deckhut et al., 1993), while the Vβ8.2+ H-$2D^b$+ Sendai $NP_{324–332}$-specific hybridoma was generated from H-2^{bm1} animals that lack the immunodominant H-$2K^b$+ $NP_{324–332}$ response (Cole et al., 1997).

Flow Cytometry and FACS Analysis

Single cell suspensions of lymphocytes were blocked in purified anti-mouse CD16/CD32 (Fc-γIII/II receptor, Pharmingen, San Diego, CA), then stained with the NPP and SEV9 tetramers for 1 hr at room temperature. The two- or three-color flow cytometric analysis (Tripp et al., 1995a) utilized various combinations of FITC-conjugated or biotinylated MAbs (all supplied by Pharmingen) specific for CD8 (53–6.7), CD62L (MEL-14), or CD44 (IM7), and a range of TCR Vβ and Vα chains (Deckhut et al., 1993; Cole et al., 1994). Samples were acquired on a FACScan flow cytometer, and the data was analyzed using CELLQuest software (Becton-Dickinson Immunocytometry Systems, San Jose, CA).

Intracellular IFNγ Staining Following Peptide Stimulation

The cell populations (5–8 $\times$ 10^5 in 200 μl of complete medium with 10 μg/ml Brefeldin A) were cultured for 6 hr in 96-well U-bottomed plates in the presence or absence of 1 μM viral peptides (Murali-Krishna et al., 1998). The viral peptides were the $NP_{366–374}$ used also to make the NPP tetramer and the subdominant $NS2_{114–121}$ that binds H-$2K^b$. As of yet, no tetrameric complexes have been made with H-$2K^b$. After culture, the responder cells were washed twice in PBS/Brefeldin A (10 μg/ml), blocked with the MAb to FcγIII/II and stained with rat anti-mouse CD8-FITC MAb (Pharmingen). They were then washed again in PBS/Brefeldin A, fixed in 1% formaldehyde in PBS for 20 min, washed in PBS, placed in PBS/0.5% Saponin (Sigma, St Louis, MO) for 10 min, and incubated with a rat anti-mouse IFNγ-PE MAb or a rat IgG1-PE control MAb. The specificity of staining was confirmed in separate control experiments (data not shown) by blocking with excess recombinant IFNγ.

Acknowledgments

The NPP and SEV9 tetramers were made with the help of Joe Miller. Edward Usherwood provided the SEV9 tetramer. We thank Sherri Surman and Kristen Branum for technical assistance, Vicki Henderson for help with the manuscript, and Ann-Marie Hamilton-Easton for advice on flow cytometry. The panel of TCR-specific MAbs is maintained in the laboratory of Marcy Blackman. Public Health Service grants AI29579, AI38359, AI37597, and CA21765, and the American Lebanese Syrian Associated Charities supported these experiments. G. T. B. is a C. J. Martin fellow of the Australian National Health and Medical Research Council (Reg. Key. 977 309).

Received April 9, 1998; revised May 8, 1998.

References

Ahmed, R., King, C.C., and Oldstone, M.B. (1987). Virus-lymphocyte interaction: T cells of the helper subset are infected with lymphocytic choriomeningitis virus during persistent infection in vivo. J. Virol. *61*, 1571–1576.

Ahmed, R., and Gray, D. (1996). Immunological memory and protective immunity: their relation. Science *272*, 54–60.

Allan, W., Tabi, Z., Cleary, A., and Doherty, P.C. (1990). Cellular events in the lymph node and lung of mice with influenza. Consequences of depleting $CD4^+$ T cells. J. Immunol. *144*, 3980–3986.

Allan, W., Carding, S.R., Eichelberger, M., and Doherty, P.C. (1993). hsp65 mRNA+ macrophages and $\gamma\delta$ T cells in influenza virus-infected mice depleted of the $CD4^+$ and $CD8^+$ lymphocyte subsets. Microb. Pathog. *14*, 75–84.

Allouche, M., Owen, J.A., and Doherty, P.C. (1982). Limit-dilution analysis of weak influenza-immune T cell responses associated with H-$2K^b$ and H-$2D^b$. J. Immunol. *129*, 689–693.

Altman, J.D., Moss, P.A.H., Goulder, P.J.R., Barouch, D.H., McHeyzer-Williams, M.G., Bell, J.I., McMichael, A.J., and Davis, M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science *274*, 94–96.

Andrew, M.E., Coupar, B.E., Boyle, D.B., and Ada, G.L. (1987). The roles of influenza virus haemagglutinin and nucleoprotein in protection: analysis using vaccinia virus recombinants. Scand. J. Immunol. *25*, 21–28.

Askonas, B.A., Mullbacher, A., and Ashman, R.B. (1982). Cytotoxic T-memory cells in virus infection and the specificity of helper T cells. Immunology *45*, 79–84.

Bender, B.S., Taylor, S.F., Zander, D.S., and Cottey, R. (1995). Pulmonary response of young and aged mice after influenza virus challenge. J. Lab. Clin. Invest. *126*, 169–177.

Bennink, J., Effros, R.B., and Doherty, P.C. (1978). Influenza pneumonia: early appearance of cross-reactive T cells in lungs of mice primed with heterologous type A viruses. Immunol. *35*, 503–509.

Buchmeier, M.J., Welsh, R.M., Dutko, F.J., and Oldstone, M.B. (1980). The virology and immunobiology of lymphocytic choriomeningitis virus infection. Adv. Immunol. *30*, 275–331.

Busch, D.H., Pilip, I.M., Vijh, S., and Pamer, E.G. (1998). Coordinate regulation of complex T cell populations responding to bacterial infection. Immunity *8*, 353–362.

Butcher, E.C., and Picker, L.J. (1996). Lymphocyte homing and homeostasis. Science *272*, 60–66.

Butz, E.A., and Bevan, M.J. (1998). Massive expansion of antigen-specific $CD8^+$ T cells during an acute virus infection. Immunity *8*, 167–175.

Cole, G.A., Katz, J.M., Hogg, T.L., Ryan, K.W., Portner, A., and Woodland, D.L. (1994). Analysis of the primary T-cell response to Sendai virus infection in C57BL/6 mice: $CD4^+$ T-cell recognition is directed predominantly to the hemagglutinin-neuraminidase glycoprotein. J. Virol. *68*, 6863–6870.

Cole, G.A., Hogg, T.L., Coppola, M.A., and Woodland, D.L. (1997). Efficient priming of $CD8^+$ memory T cells specific for a subdominant epitope following Sendai virus infection. J. Immunol. *158*, 4301–4309.

Deckhut, A.M, Allan, W., McMickle, A., Eichelberger, M., Blackman, M.A., Doherty, P.C., and Woodland, D.L. (1993). Prominent use of Vβ8.3 T cells in the H-$2D^b$ restricted response to an influenza A virus nucleoprotein epitope. J. Immunol. *151*, 2658–2666.

DeGrendele, H.C., Estess, P., and Siegelman, M.H. (1997). Requirement for CD44 in activated T cell extravasation into an inflammatory site. Science *278*, 672–675.

Doherty, P.C., Biddison, W.E., Bennink, J.R., and Knowles, B.B. (1978). Cytotoxic T-cell responses in mice infected with influenza and vaccinia viruses vary in magnitude with H-2 genotype. J. Exp. Med. *148*, 534–543.

Doherty, P.C., Allan, W., Boyle, D.B., Coupar, B.E., and Andrew, M.E. (1989). Recombinant vaccinia viruses and the development of immunization strategies using influenza virus. J. Infect. Dis. *159*, 1119–1122.

Doherty, P.C., Allan, W., Eichelberger, M., and Carding, S.R. (1992). Roles of αβ and γδ T cell subsets in viral immunity. Annu. Rev. Immunol. *10*, 123–151.

Doherty, P.C., Topham, D.J., and Tripp, R.A. (1996). Establishment and persistence of virus-specific CD4+ and CD8+ T cell memory. Immunol. Rev. *150*, 23–44.

Eichelberger, M.C., Wang, M., Allan, W., Webster, R.G., and Doherty, P.C. (1991). Influenza virus RNA in the lung and lymphoid tissue of immunologically intact and CD4-depleted mice. J. Gen. Virol. *72*, 1695–1698.

Gresser, I., Guy-Grand, D., Maury, C., and Maunoury, M.T. (1981). Interferon induces peripheral lymphadenopathy in mice. J. Immunol. *127*, 1569–1575.

Hamilton-Easton, A., and Eichelberger, M. (1995). Virus-specific antigen presentation by different subsets of cells from lung and mediastinal lymph node tissues of influenza virus-infected mice. J. Virol. *69*, 6359–6366.

Holt, P.G., Oliver, J., Bilyk, N., McMenamin, C., McMenamin, P.G., Kraal, G., and Thepen, T. (1993). Downregulation of the antigen presenting cell function(s) of pulmonary dendritic cells in vivo by resident alveolar macrophages. J. Exp. Med. *177*, 397–407.

Horimoto, T., and Kawaoka, Y. (1995). The hemagglutinin cleavability of a virulent avian influenza virus by subtilisin-like endoproteases is influenced by the amino acid immediately downstream of the cleavage site. Virology *210*, 466–470.

Hou, S., and Doherty, P.C. (1993). Partitioning of responder CD8+ T cells in lymph node and lung of mice with Sendai virus pneumonia by LECAM-1 and CD45RB phenotype. J. Immunol. *150*, 5494–5500.

Hou, S., Doherty, P.C., Zijlstra, M., Jaenisch, R., and Katz, J.M. (1992). Delayed clearance of Sendai virus in mice lacking class I MHC-restricted CD8+ T cells. J. Immunol. *149*, 1319–1325.

Hou, S., Hyland, L., Ryan, K.W., Portner, A., and Doherty, P.C. (1994). Virus-specific CD8+ T-cell memory determined by clonal burst size. Nature *369*, 652–654.

Iezzi, G., Karajalainen, K., and Lanzevecchia, A. (1998). The duration of antigenic stimulation determines the fate of naive and effector T cells. Immunity *8*, 89–95.

Kast, W.M., Roux, L., Curren, J., Blom, J., Vorrdrouw, A.C., Meloen, R.H., Kolakofsky, D., and Melief, C.J. (1991). Protection against lethal Sendai virus infection by in vivo priming virus-specific cytotoxic T lymphocytes with a free synthetic peptide. Proc. Natl. Acad. Sci. USA *88*, 2283–2287.

Kilbourne, E.D. (1969). Future influenza vaccines and the use of genetic recombinants. Bull. World Health Organ. *41*, 643–645.

Kitamura, D., Roes, J., Kuhn, R., and Rajewsky, K. (1991). A B cell-deficient mouse by targeted disruption of the membrane exon of the immunoglobulin μ-chain gene. Nature *350*, 423–426.

Klenerman, P., Hengartner, H., and Zinkernagel, R.M. (1997). A non-retroviral RNA virus persists in DNA form. Nature *390*, 298–301.

Korngold, R., Blank, K.J., and Murasko, D.M. (1983). Effect of interferon on thoracicduct lymphocyte output: induction with either poly I:poly C or vaccinia virus. J. Immunol. *130*, 2236–2240.

Lalvani, A., Brookes, R., Hambleton, S., Britton, W.J., Hill, A.V.S., and McMichael, A.J. (1997). Rapid effector function in CD8+ memory T cells. J. Exp. Med. *186*, 859–865.

Lau, L.L., Jamieson, B.D., Somasundaram, T., and Ahmed, R. (1994). Cytotoxic T-cell memory without antigen. Nature *369*, 648–652.

Lawson, C.M., Bennink, J.R., Restifo, N.P., Yewdell, J.W., and Murphy, B.R. (1994). Primary pulmonary cytotoxic T lymphocytes induced by immunization with a vaccinia virus recombinant expressing influenza A virus nucleoprotein peptide do not protect mice against challenge. J. Virol. *68*, 3505–3511.

Lynch, F., Doherty, P.C., and Ceredig, R. (1989). Phenotypic and functional analysis of the cellular response in regional lymphoid tissue during an acute virus infection. J. Immunol. *142*, 3592–3598.

Mackay, C.R., Marston, W.L., Dudler, L., Spertini, O., Tedder, T.F., and Hein, W.R. (1992). Tissue-specific migration pathways by phenotypically distinct subpopulations of memory T cells. Eur. J. Immunol. *22*, 887–895.

Matzinger, P. (1994). Tolerance, danger, and the extended family. Annu. Rev. Immunol. *12*, 991–1045.

McHeyzer-Williams, M.G., Altman, J.D., and Davis, M.M. (1996). Tracking antigen-specific helper T cell responses. Curr. Opin. Immunol. *8*, 278–284.

McWilliam, A.S., Marsh, A.M., and Holt, P.G. (1997). Inflammatory infiltration of the upper airway epithelium during Sendai virus infection: involvement of epithelial dendritic cells. J. Virol. *71*, 226–236.

Mullbacher, A. (1994). The long-term maintenance of cytotoxic T cell memory does not require persistence of antigen. J. Exp. Med. *179*, 317–321.

Murali-Krishna, K., Altman, J.D., Suresh, M., Sourdive, D., Zajac, A., Miller, J., Slansky, J., and Ahmed, R. (1998). Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity *8*, 177–187.

Topham, D.J., and Doherty, P.C. (1998). Clearance of an influenza A virus by CD4+ T cells is inefficient in the absence of B cells. J. Virol. *72*, 882–885.

Topham, D.J., Tripp, R.A., and Doherty, P.C. (1997). CD8+ T cells clear influenza virus by perforin or Fas-dependent processes. J. Immunol. *159*, 5197–5200.

Tough, D.F., Borrow, P., and Sprent, J. (1996). Induction of bystander T cell proliferation by viruses and type I interferon *in vivo*. Science *272*, 1947–1950.

Townsend, A.R., Rothbard, J., Gotch, F.M., Bahadur, G., Wraith, D., and McMichael, A.J. (1986). The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell *44*, 959–968.

Tripp, R.A., Hou, S., and Doherty, P.C. (1995a). Temporal loss of the activated L-selectin-low phenotype for virus-specific CD8+ memory T cells. J. Immunol. *154*, 5870–5875.

Tripp, R.A., Hou, S., McMickle, A., Houston, J., and Doherty, P.C. (1995b). Recruitment and proliferation of CD8+ T cells in respiratory virus infections. J. Immunol. *154*, 6013–6021.

Tripp, R.A., Lahti, J.M., and Doherty, P.C. (1995c). Laser light suicide of proliferating virus-specific CD8+ T cells in an *in vivo* response. J. Immunol. *155*, 3719–3721.

Vitiello, A., Yuan, L., Chesnut, R.W., Sidney, J., Southwood, S., Farness, P., Jackson, M.R., Peterson, P.A., and Sette, A. (1996). Immunodominance analysis of CTL responses to influenza PR8 virus reveals two new dominant and subdominant Kb-restricted epitopes. J. Immunol. *157*, 5555–5562.

Walker, J.A., Sakaguchi, T., Matsuda, Y., Yoshida, T., and Kawaoka, Y. (1992). Location and character of the cellular enzyme that cleaves the hemagglutinin of a virulent avian influenza virus. Virology *190*, 278–287.

Zinkernagel, R.M., and Doherty, P.C. (1973). Cytotoxic thymus-derived lymphocytes in cerebrospinal fluid of mice with lymphocytic choriomeningitis. J. Exp. Med. *138*, 1266–1269.

Zinkernagel, R.M., and Doherty, P.C. (1974). Restriction of in vitro T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. Nature *248*, 701–702.