Quantification of Repertoire Diversity of Influenza-Specific Epitopes with Predominant Public or Private TCR Usage[1]

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The H-2D^b-restricted CD8 T cell immune response to influenza A is directed at two well-described epitopes, nucleoprotein 366 (NP366) and acid polymerase 224 (PA224). The responses to the two epitopes are very different. The epitope NP366-specific response is dominated by TCR clonotypes that are public (shared by most mice), whereas the epitope PA224-specific response is private (unique within each infected animal). In addition to being public, the NP366-specific response is dominated by a few clonotypes, when T cell clonotypes expressing the Vβ8.3 element are analyzed. Herein, we show that this response is similarly public when the NP366$^+$Vβ4$^+$ CD8 T cell response is analyzed. Furthermore, to determine whether these features resulted in differences in total TCR diversity in the NP366$^+$ and PA224$^+$ responses, we quantified the number of different CD8 T clonotypes responding to each epitope. We calculated that 50–550 clonotypes recognized each epitope in individual mice. Thus, although the character of the response to the two epitopes appeared to be different (private and diverse vs public and dominated by a few clonotypes), similar numbers of precursor cells responded to both epitopes and this number was of similar magnitude to that previously reported for other viral CD8 T cell epitopes. Therefore, even in CD8 T cell responses that appear to be oligoclonotypic, the total response is highly diverse. *The Journal of Immunology,* **2006, 177: 6705–6712.**

Cytotoxic CD8 T cells, critical for intracellular pathogen clearance, recognize MHC class I/peptide complexes displayed on the surface of infected cells. After encounter with Ag, epitope-specific T cells proliferate and differentiate in a programmed fashion. The diversity (and specificity) of the response primarily results from imprecise joining of the V, D, and J regions of the α- and β-chains in the CDR3 of the TCR. Several studies of TCR diversity suggested that the CD8 T cell response was oligoclonal (1–3). These studies used model Ag systems or infusion of TCR transgenic CD8 T cells into virus-infected mice to calculate TCR diversity. However, other studies, in which TCR diversity after viral infection was determined, suggested that the response was highly diverse, with estimates of several hundred different clonotypes responding to a single epitope (4–8).

The naive mouse contains $\sim$2–4 $\times$ 10^7 CD8 T cells, with an estimated diversity of 2–5 $\times$ 10^5 different clonotypes per spleen (9, 10). If many clonotypes respond to a single epitope, a single TCR will need to recognize more than one epitope, to facilitate recognition of a large and diverse population of Ags (11). Consistent with this prediction, recent studies have demonstrated the existence of heterologous immunity—the reactivation of memory cells in response to infection with a second pathogen (8, 9). CD8 T cells responding to an epitope exhibit both public (TCRs shared among most individuals) and private (TCRs unique to individual animals) specificities, but the cross-reacting cells are predominantly from the private group of TCRs. As a consequence, individual animals (including humans) will exhibit different patterns of heterologous immunity (12).

CD8 T cell responses have been extensively studied in C57BL/6 (B6) mice infected with influenza A virus (13, 14). Primary respiratory disease is generally induced by intranasal infection of naive B6 mice with the A/HKx31 (H3N2) influenza A virus. After the virus is cleared (around day 10) and memory CD8 T cell populations are established (4–6 wk), a secondary CD8 T cell response is elicited by infection with a heterologous influenza strain such as A/PR/34 (H1N1) (H1N1 prime/H3N2 challenge). The PR8 and HKx31 viruses share six internal gene products (including nucleoprotein (NP)[3] and acid polymerase (PA), which give rise to the principal epitopes recognized by CD8 T cells) but express distinct surface hemagglutinin and neuraminidase proteins. Hence, prime/challenge experiments avoid the complication of reduced virus doses due to Ab-mediated neutralization of the second inoculum. Numerically, the most prominent CD8 T cell subsets are specific for two epitopes derived from the viral nucleoprotein (NP366–374) and acid polymerase (PA224–233) proteins (15, 16). Following primary infection the NP366$^+$ and PA224$^+$ CD8 T cell responses are essentially codominant (approximately equivalent magnitude), although the PA224$^+$ CD8 T cell response peaks 1–2 days earlier (17). However, following secondary challenge, the NP366$^+$ CD8 T cell population is clearly immunodominant, constituting up to 80% of the total virus-specific CD8 T cell response and ranging from 5- to 10-fold greater in magnitude than the next largest PA224$^+$ response (16, 18). The T cell responses to both

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[3] Abbreviations used in this paper: NP, nucleoprotein; PA, acid polymerase; LCMV, lymphocytic choriomeningitis virus; MHV, mouse hepatitis virus.

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epitopes have been studied extensively. Strikingly, the predominant responses to epitope NP366 exhibited largely public specificity, whereas the response to epitope PA224 was largely unique in each infected animal (19–21). The NP366$^+$ response showed preferential usage of the Vβ8.3 element (~30%), whereas the Vβ7 element was overrepresented in the population of CD8 T cells responding to epitope PA224 (22, 23). However, as in mice infected with either lymphocytic choriomeningitis virus (LCMV) or mouse hepatitis virus (MHV), the influenza-specific TCR repertoire included clonotypes that were abundant as well as others that were present at low frequency.

Previously, we showed that the numbers of clonotypes in individual mice responding to a single viral epitope were well described by a log-series, and from these distributions, we calculated the number of different epitope-specific clonotypes in the CNS and spleen of animals infected with MHV or LCMV (4, 5). Because the Vβ8.3$^+$ CD8 T cell response to epitope NP336 was unusual in containing preferential expansion of a limited set of public clonotypes, we postulated that this response would comprise a smaller repertoire than was observed in LCMV- or MHV-infected animals. Herein, we quantified the diversity of the epitope NP366- and PA224-specific CD8 T cell responses. We also investigated whether the limited, public response manifested by NP366$^+$Vβ8.3$^+$ CD8 T cells was present when NP366$^+$ cells expressing another Vβ element were analyzed.

Materials and Methods

Mice and virus infections

Female C56BL/6J (H-2^b) mice were bred at the University of Melbourne (Parkville, Australia). Some were anesthetized at 6 wk of age by isoflurane inhalation and infected intranasally with 10^4 PFU of the HKx31 (H3N2) influenza A virus in 30 μl of PBS. Memory mice for secondary challenge experiments were injected i.p. at least 6 wk previously with 1.5×10^7 PFU of the PR8 (H1N1) influenza A virus. Both virus stocks were grown in the allantoic cavity of 10-day embryonated hen's eggs and titered on Madin-Darby canine kidney cells. All animal work was in compliance with the guidelines set out by the University of Melbourne Animal Experimental Ethics Committee.

Tissue sampling and cell preparation

Mice were anesthetized i.p. with 3 mg of ketamine and 0.6 mg of Xylazil (Parnell Laboratories). Spleens were removed, disrupted, and enriched for T cells by using plate-bound goat anti-mouse IgG and IgM Abs (Jackson ImmunoResearch).

Single-cell RT-PCR and sequencing

T cell-enriched lymphocyte populations were stained either with D^{b}NP366 (ASNENMETM)- or D^{b}PA224 (SSLENFRAYV)-specific tetramers conjugated to streptavidin-PE (Molecular Probes) for 60 min at room temperature, followed by two washes in sort buffer (0.1% BSA in PBS). Cells were then stained with anti-CD8-allophycocyanin and anti-Vβ8.3- or anti-Vβ7-FITC Abs (BD Pharmingen) for 30 min on ice and washed twice. Cells were resuspended in 500 μl of sort buffer and transferred to polypropylene FACS tubes (BD Labware) for subsequent sorting experiments. Lymphocytes were isolated using a MoFlo sorter (DakoCytomation) fitted with a "Cyclone" single-cell deposition unit under stringent conditions. Single D^{b}NP336$^+$Vβ8.3$^+$CD8$^+$ or D^{b}PA224$^+$Vβ7$^+$CD8$^+$ cells (see Fig. 1) were sorted directly into a 96-well PCR plate (Eppendorf) containing 5 μl of cDNA reaction mix. Negative controls were interspersed between the samples (1 in 10), and 80 cells were sorted per plate. The cDNA mix contained 0.25 μl of Sensiscript reverse transcriptase, 1× cDNA buffer, 0.5 mM dNTPs (Qiagen), 0.125 μg of oligo(dT) (Promega), 100 μg/ml gelatin (Roche), 100 μg/ml tRNA (Roche), 20 U RNAsin (Invitrogen Life Technologies), and 0.1% Triton X-100 (Sigma-Aldrich). After sorting, plates were incubated at 37°C for 90 min for cDNA synthesis, followed by 5 min at 95°C. Plates were stored at −80°C. The Vβ8.3$^+$ and Vβ7$^+$ transcripts were amplified and sequenced as previously described (19, 20).

Isolation of RNA from bulk populations of tetramer$^+$ lymphocytes and cDNA synthesis

Tetramer D^{b}NP366$^+$CD8$^+$ and tetramer D^{b}PA224$^+$CD8$^+$ cells were sorted into Eppendorf tubes. RNA was prepared using TRIzol (Invitrogen Life Technologies). cDNA was reverse-transcribed using an Omniscript RT kit (Qiagen) according to the manufacturer's instructions. Vβ8.3, Vβ4, Vβ7, and Vβ8.1/8.2 transcripts were amplified using the following sense primers: Vβ8.3 (5′-ACC AGA ACA ACG CAA GAA GAC-3′), Vβ7 (5′-TAC AGG GTC TCA CGG AAG AAG C-3′), Vβ4 (5′-TCA GAC TGC CTC AAG TCG CTT CC-3′), Vβ8.1/8.2 (5′-TAC AAG GCC TCC AGA CCA AGC CAA-3′), and the antisense primer Cβb (5′-CTT GGG TGG AGT CAC ATT TCT C-3′). PCR products were cloned into plasmid vector pCR2.1-TOPO, following the instructions of the manufacturer (Invitrogen Life Technologies). Colonies containing inserts were identified and CDR3 of the TCR β-chain (CDR3β) were sequenced using an Applied Biosystems Prism 3700 sequence analyzer.

TCR repertoire analysis

We fit log-series distributions to our clonotype diversity data. Log-series distributions are often used to describe data with many rare species and a few abundant ones (24, 25). The distribution is specified by the equation $s_i = \alpha x^i/i$, where s_i is the number of sequences expected to be represented i times, α is an index of clonal diversity, and x is a fitted parameter related to diversity and sample size (number of clones sequenced). The diversity index α depends on S, the number of different CDR3β sequences present in the T cell population, and N, the total number of T cells in the population: $S = \alpha \ln(1 + N/\alpha)$. When the number of different CDR3β sequences is high relative to the number of T cells, α is large. To calculate α, we used a computer program written in QuickBASIC 4.5 (Microsoft) following algorithms in Krebs (26). SEs for α were obtained following Ref. 27.

In this study, we performed additional analyses to test the suitability of log-series distributions for fitting our data using a Monte Carlo procedure (28). This procedure allowed us higher power to detect deviations from log-series than the conventional goodness-of-fit tests applied in our previous work (4, 5). We began by describing the shape of a clonotype diversity distribution by calculating an observed "tailiness" statistic, $T_o = \sum i^2 s_i$, where s_i is the number of clonotypes represented i times. "Taily" distributions (large T_o; see, e.g., Fig. 2*A*) are those with relatively more abundant and rare sequences (the tails of the frequency distribution); "nontaily" distribution (small T_o) have few abundant and rare sequences, but many sequences of intermediate frequency. We then sought to compare tailiness for an actual dataset with observed diversity α_o to the tailiness expected for a true log-series distribution with the same diversity. In principle, this could be done by simulating log-series datasets using the log-series generating function (26) with $\alpha = \alpha_o$, but a complication arises because in the theoretical distribution, extremely abundant clonotypes occur with small but nonzero probability. For real datasets, in contrast, no single clonotype can be more abundant than the total number of cells sequenced. As a result, generating a true log-series dataset using the generating function and $\alpha = \alpha_o$ produces simulated data from which our fitting procedure estimates an α that slightly exceeds α_o. This mismatch could lead our tailiness test to reject a log-series fit even when it is appropriate. As a result, we adopted a slightly more complicated procedure, in which we found for each real dataset (by iterative fitting) a corrected diversity index α_c such that true log-series datasets generated using $\alpha = \alpha_c$ yield fitted values $\alpha = \alpha_o$. We then generate 10,000 true log-series distributions (with the same total number of individuals cells and using $\alpha = \alpha_c$), calculate tailiness (T_{ls}) for each, and compare the observed tailiness T_o to distribution of log-series T values. The fraction of log-series T_{ls} values $\geq T_o$ is then a (one-sided) p value for the test of fit of the observed data to log-series (for data that fit a log-series perfectly, we find $p = 0.5$, confirming the appropriateness of our procedure). Finally, we calculated a relative tailiness T_R for each dataset, where $T_R = T_o/\text{mean}(T_{ls})$ and $T_R = 1$ for data that fit a log-series perfectly.

For each mouse dataset, we calculated the approximate number of Vβ8.3 and Vβ4 NP366-specific and Vβ7 and Vβ8.1/2 PA224-specific CD8 T clonotypes within the spleen, and the expected number per 10,000 cells. For datasets that were too taily to fit the log-series well (too many rare and common sequences; T_o with $p < 0.05$), we provide both estimates ignoring the lack of fit and estimates correcting for it. Ignoring the lack of fit leads to an underestimate of true diversity (because it ignores some of the rare sequences). To provide a corrected estimate, we eliminated the most common sequences in turn until the remaining distribution fit a log-series (see Table IV), and estimated diversity via a combination of two components: a small set of one to three superabundant clonotypes whose abundance could be directly counted, and a much larger set of less abundant clonotypes whose frequencies and diversity were estimated from a

fitted log-series distribution. This correction should be seen as tentative, because for some datasets removal of the most abundant sequences left relatively few observed clonotypes (<30), making the log-series fit somewhat uncertain. However, all of our major conclusions are robust to the difference between uncorrected and corrected diversity estimates.

We compared diversity estimates among groups of datasets (e.g., primary vs secondary responses) with two-sample *t* tests. Because diversity estimates are unlikely to have normal distributions, we determined *p* values for these tests by randomization (28) using a computer program written in QuickBASIC 4.5 (Microsoft).

Results

Diversity of epitope NP366$^+$Vβ4$^+$ CD8 T cells

In our initial studies of the diversity of the epitope NP366-specific response, we performed RT-PCR analyses on single NP366$^+$Vβ8.3$^+$CD8$^+$ T cells. Based on analysis of these cells, we concluded that the epitope NP366$^+$ response was dominated by public sequences (TCRs shared by most individuals). To determine whether this preferential public response was unique to NP366$^+$Vβ8.3$^+$ CD8 T cells, we examined the diversity of cells expressing a second Vβ element. CD8 T cells expressing the Vβ4 element comprise ~13% of NP366$^+$ cells and were chosen for further study. However, the relatively small proportion of NP366$^+$Vβ4$^+$ CD8 T cells precluded this approach. Therefore, before studies of NP366$^+$Vβ4$^+$ cells, we determined whether diversity analyses of single cell and bulk cDNA populations gave similar results, using mice after secondary infection. Splenic samples were chosen because of ease of analysis and previously, we showed that influenza-specific CD8 T cell clonotypic distributions were the same in the spleen and the lung, the site of infection (20). Splenocytes from mice undergoing a secondary immune response were used in these analyses, to increase the number of virus-specific T cells (18).

We identified NP366$^+$ and PA224$^+$ CD8 T cells from infected spleens by staining with MHC class I/peptide tetramer and sorting with a flow cytometer (Fig. 1, *upper panels*). cDNA was developed from this population (bulk cDNA). Some cells were also stained for Vβ8.3 (NP366$^+$) or Vβ7 (PA224$^+$) before sorting and used in single-cell RT-PCR analyses (single cell cDNA; Fig. 1, *lower panels*) (19). We then sequenced individual cDNA clones, as described previously (19). Bulk and single-cell NP366$^+$Vβ8.3$^+$ or PA224$^+$Vβ7$^+$ CD8 T cells splenic populations from three and two mice, respectively, were analyzed. Distributions of individual CDR3β sequences obtained by bulk and single-cell analyses were indistinguishable for two NP366$^+$Vβ8.3$^+$ and two PA224$^+$Vβ7$^+$ CD8 T cell samples, as illustrated in Table I for a representative mouse (mouse 5, *G* test, $G = 0.43$, 3 d.f., $p = 0.93$). As anticipated, some lower frequency sequences were detected in only one population (e.g., SDVISTEV was detected in analyses of bulk cDNA but not single-cell cDNA). One of the five mice (mouse no. 7, Table I) did show differences in clonotype frequency distribution between methods ($G = 39.1$, 5 df, $p < 0.0001$), with more singletons detected in the bulk cDNA analysis. However, none of our major conclusions would be affected if differences of this magnitude occurred in some mice.

Next, we analyzed the Vβ4$^+$ populations from the same NP366$^+$ bulk cDNA secondary effector populations described above and from three mice after primary influenza infection (Table II). Only a small number of Vβ4$^+$ cDNA clones were obtained from the primary effector populations and these exhibited limited diversity. However, the majority of CDR3β in every mouse either contained a common motif SQDRR and were Jβ1.6$^+$ or were from a single Jβ2.3$^+$ clonotype (CDR3β: SQDNRG). Private sequences, unique to individual mice, were also identified in all mice after secondary infection but were not detected to a significant extent after primary infection. A lower diversity in the primary as opposed to secondary response is not expected (e.g., Refs. 29 and 30), but clonotypes detected in secondary but not primary influenza A CD8 T cell responses have been reported previously (19,

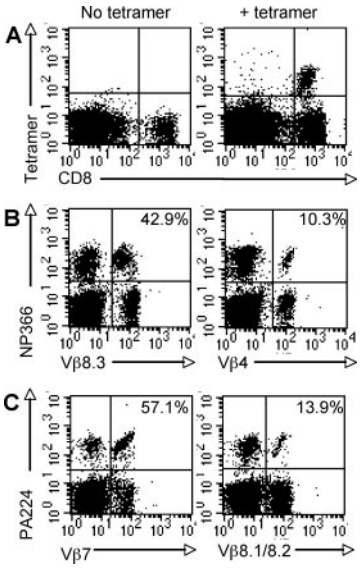

FIGURE 1. Dominant and subdominant Vβ usage for D^{b}NP366$^+$CD8$^+$ and D^{b}PA224$^+$CD8$^+$ T cell responses. Splenocytes were obtained from mice infected intranasally with HKx31 virus, enriched for CD8 T cells, and stained with D^{b}NP366- or D^{b}PA224-specific tetramers conjugated to streptavidin-PE and anti-CD8-allophycocyanin and anti-Vβ-FITC mAbs. *A*, Representative dot plots from analyses performed at the acute phase (day 11 postinfection) are shown after CD8 and tetramer staining. *B*, Vβ8.3 (dominant) and Vβ4 (subdominant) staining of NP366$^+$ CD8 T cells is shown. *C*, Vβ7 (dominant) and Vβ8.1/8.2 (subdominant) staining of PA224$^+$ CD8 T cells is shown. *B* and *C*, Percentage of Vβ usage of tetramer$^+$ CD8 T cells is displayed in the *top right quadrant*. Cells were gated on CD8 T cells.

Table I. *Comparison of NP366$^+$Vβ8.3$^+$ CDR3β sequences derived from single-cell and bulk cDNA populations*

Mouse	CDR3β Loop	Jβ	Frequency (%)	
			Single cell ($n = 55$)	Bulk ($n = 78$)
No. 5	RGGANTGQL	2.2	21/55 (38%)	30/82 (36.6%)
	SGGSNTGQL	2.2	21/55 (38%)	29/82 (35.4%)
	SDAISTEV	1.1	8/55 (14.5%)	13/82 (15.9%)
	SDVISTEV	1.1		6/82 (7.3%)
	SGGANTGQL	2.2	2/55 (3.6%)	2/82 (2.4%)
	RGGSNTGQL	2.2	2/55 (3.6%)	
	SVGGRTTQ	2.5		1/82 (1.2%)
	SDASKTEV	1.1	1/55 (1.8%)	
	SAGGRDTQ	2.5		1/82 (1.2%)
No. 7	SGGGNTGQL	2.2	23/66 (34.8%)	21/78 (27%)
	SGGSNTGQL	2.2	5/66 (7.6%)	29/78 (37%)
	SGGANTGQL	2.2	14/66 (21%)	8/78 (10%)
	RGGGNTGQL	2.2	11/66 (16.7%)	2/78 (2.6%)
	KGGANTGQL	2.2	10/66 (15%)	3/78 (3.8%)
	SDAAGPEQ	2.6	1/66 (1.5%)	6/78 (1.3%)
	SAGGRNTL	2.4	1/66 (1.5%)	1/78 (1.3%)
	KAGGNTGQL	2.2		2/78 (2.6%)
	SPAKPVAET	2.3	1/66 (1.5%)	
	SDARTTEV	1.1		1/78 (1.3%)
	SEETNTEV	1.1		1/78 (1.3%)
	SEVRGDTQ	2.5		1/78 (1.3%)
	SDAANTEV	1.1		1/78 (1.3%)
	SEGARTEQ	2.6		1/78 (1.3%)
	RGGANTGQL	2.2		1/78 (1.3%)

Table II. *Frequency of NP366$^+$Vβ4$^+$ CDR3β sequences*

CDR3β Loop	Jβ	Mouse (1° or 2° response)					
		No. 12 (1°)	No. 13 (1°)	No. 14 (1°)	No. 5 (2°)	No. 6 (2°)	No. 7 (2°)
SQDNRGSAETL	2.3				33/58	6/64	16/66
SQDRRPSYNSPL	1.6	4/7	35/35	11/20	6/58	2/64	
SQDRRSYNSPL	1.6					34/64	
SQERGSAETL	2.3	3/7					
SQESGVGERL	1.4			8/20			
SQESGVGERS	1.4			1/20			
SQGTGVNSPL	1.6				6/58	4/64	2/66
RSTVNQDTQ	2.5				2/58		
RQGREQ	2.6				2/58		
SQDRRNSYNSPL	1.6				1/58	4/64	20/66
SRDRRPSYNSPL	1.6				1/58		
SRDWGGSQNTL	2.4				1/58		
SPDRGGDTQ	2.5				1/58		
SQDRRGSYNSPL	1.6				1/58	12/64	1/66
SQDPGQKNTL	2.4				1/58		
SQERAGNYAEQ	2.1				1/58		
SQENSYNSPL	1.6				1/58		
SRTDYAEQ	2.1				1/58		
SQDLRSSYNSPL	1.6					1/64	
SPDRGGDTN	2.5					1/64	
SQDRDRTYEQ	2.6						10/66
SQDRRSSYNSPL	1.6						7/66
SQDEGAVTEV	1.1						6/66
DQDLPGKAEG	2.1						1/66
GQDGGRTYEQ	2.6						1/66
SPGNWGSAETL	2.3						1/66
SQGDWGSAETL	2.3						1/66

20). This low diversity precluded any further analysis of the NP366$^+$Vβ4$^+$ primary response.

Quantification of diversity of epitope NP366$^+$ CD8 T cells

These results, as well those of previous studies, suggested that the epitope NP366$^+$ response exhibited limited diversity, when compared with the TCR diversity of cells responding to MHV CD8 T cell epitope S510 and LCMV CD8 T cell epitope gp33 (4, 5). Because the NP366$^+$ responses were dominated by a few CDR3β clonotypes that were detected in most mice, the fraction of private sequences was also lower than for the S510 and gp33 epitopes (4, 5). The TCRβ response to the latter two epitopes can be described by a log-series distribution. To quantify epitope NP366-specific diversity, we examined whether the response to this epitope could also be described by a log-series. For these studies, we analyzed four naive mice infected intranasally with influenza X31 (primary infection) and seven influenza X31-immune mice with PR8 virus including the three described in Fig. 1 (secondary response). As above, epitope-specific CD8 T cells were identified by D^{b}NP366$^+$ tetramer staining and Vβ8.3 and Vβ4-encoding cDNA clones were prepared. CDR3β sequence analysis was used to determine the frequency of each clonotype within the epitope NP366-specific population (Table III). In some instances, distinct nucleotide sequences encoded the same CDR3β and these were counted as separate clonotypes. The data were then fit to a logarithmic distribution as described in *Materials and Methods* (Table IV). A value of α, a measure of CDR3β diversity, was calculated for each sample. From this value, distributions of observed and expected abundance of TCR Vβ8.3 and Vβ4 cDNA clonotypes were calculated (Fig. 2*A*).

Consistent with analyses of other CD8 T cell epitopes (4, 5), the logarithmic distribution generally underestimated the number of unique sequences and the number of very common sequences within the epitope NP366-specific populations (that is, distributions were "tailier" than log-series; $T_R > 1$, illustrated in Fig. 2*A*). For many of our samples, this excess tailiness was statistically significant ($p < 0.05$), and so these samples were considered not to be well described by a log-series distribution (even though they appeared consistent with log-series in our earlier analyses, which used a less powerful test of tailiness). For these samples, we implemented the tailiness-correction procedure described in *Materials and Methods* (*right side* of Table IV). This correction modestly increased the estimated diversity of the NP366$^+$ response in each instance, but our major conclusions are robust to the treatment of taily distributions.

We used our log-series fits to estimate numbers of responding clonotypes per whole spleen, beginning with estimates of the number of NP366$^+$Vβ8.3$^+$ and NP366$^+$Vβ4$^+$ CD8 T cell clonotypes. We calculated that there were 2.6×10^5 or 1.4×10^6 NP366$^+$Vβ8.3$^+$ CD8 T cells in the spleen in the primary or secondary immune responses, respectively (primary: 9.3×10^7 cells/spleen $\times$ 10.3% CD8 T cells $\times$ 8.74% NP366$^+$ $\times$ 30.9% Vβ8.3$^+$ ($\pm$4.2% (SE), $n = 8$); secondary: 9.3×10^7 cells/spleen $\times$ 17.9% CD8 T cells $\times$ 27.8% NP366$^+$ $\times$ 30.9% Vβ8.3$^+$). The Vβ4 element was used by 13.4% ($\pm$3.1%, $n = 3$) of NP366$^+$ CD8 T cells, resulting in 6.2×10^5 cells in the secondary response. Using these estimates of cell numbers in mouse spleens and our fitted clonotype diversity indices (α), we estimated total numbers of VB8.3$^+$ TCRβ clonotypes responding to epitope NP366 in each mouse, for both primary and secondary responses. These estimates ranged among mice from 16 to 90 clonotypes. Such substantial interindividual variation appears typical of TCRβ response data (Table III, and see also Ref. 31), although we do not know what genetic or environmental factors generate it. There was no significant difference in TCRβ diversity between primary and secondary responses (for α, $p = 0.28$; for numbers of clonotypes, $p = 0.45$). Although we cannot rule out the existence of a small diversity difference between primary and secondary responses (masked by

Table III. *Diversity and abundance of epitope-specific CD8$^+$ T cells*

Mouse No.	Number of Sequences							No. Analyzed	No. Species
	1	2	3	4	5–7	8–15	16+		
Epitope NP366									
Vβ8.3 (1°)[a]									
1	3	1	2		1	1	1	60	9
2	9	1	2	1	1	3		64	17
3	3	1		1		1	2	61	8
4	4	1		1	1	1	1	61	9
Vβ8.3 (2°)									
5	5	4			2		3	137	14
6	6				2	1	3	134	12
7	10	2		2	1	4	2	144	21
8	4	1		1	2	1	1	62	10
9	2	1		1		4	2	117	10
10	1	2			3	1	1	104	8
11		4	1				1	37	6
Vβ4 (1°)									
12			1	1				7	2
13							1	35	1
14	1					2		20	3
Vβ4 (2°)									
5	9	2			2		1	58	14
6	3	1	1	3	1		1	64	10
7	7	1			2		2	66	12
Epitope PA224									
Vβ7 (1°)									
15	8	1	4	1	1	2		49	17
16	6	3	1		2	1		39	13
Vβ7 (2°)									
5	10	1	1		3	2	1	115	18
6	9	6	1	3	2	1	3	128	25
17	16	3	2		1	2		59	24
18	12	5	1	2	2	1		55	23
19	13	3	1	1		2		54	20
Vβ8.1/2 (1°)									
20	5	1	2		1			20	9
21	3		1			1		17	5
22	7					2		30	9

[a] Vβ element for primary (1°) or secondary (2°) response.

interindividual variation), any such difference is probably minor in comparison to the variation we see among mice even within a highly homogeneous group of individual animals (mice of the same strain reared under identical laboratory conditions). We presume that wild-type animals would display even more variation among individuals, although we know of no data directly addressing this presumption. Corrections for excessive numbers of common sequences led to small increases in our estimates of the number of responding clonotypes (Table IV, *right three columns*).

Similar analyses showed that 40–70 different TCRβ clonotypes were present in the NP366$^+$Vβ4$^+$ secondary response before correction for excessive numbers of common sequences (Table IV); correcting for common sequences increased our estimates of numbers of clonotypes. The Vβ8.3$^+$ and Vβ4$^+$ repertoires did not differ in α or in numbers of responding clonotypes ($p = 0.65$ and $p = 0.39$, respectively).

Assuming that the same diversity is present within each Vβ population, we can calculate the total number of NP366$^+$ clonotypes. Based on the Vβ8.3$^+$ population, there are ~50–300 NP366$^+$ CD8 T cells expressing different TCR β-chains per spleen. The Vβ4$^+$ subpopulation exhibited similar diversity, but because it represented only 13.4% of the total epitope NP366 population, the estimate of total diversity was approximately two times larger. Thus, even a CD8 T cell response dominated in each animal by a few clonotypes exhibits substantial diversity.

Quantification of diversity of PA224$^+$ CD8 T cells

The PA224$^+$ CD8 T cell response exhibits a Vβ7 bias and is largely private. On a population basis, it is very diverse (20). To determine the diversity of the response in individual mice, we developed cDNA clones from bulk populations of tetramer PA224$^+$ CD8 T cells or from single tetramer PA224$^+$Vβ7$^+$CD8$^+$ cells harvested from mice after primary or secondary influenza A infection. We also analyzed the PA224$^+$Vβ8.1/2$^+$ response in mice after primary X31 infection, to determine whether a diverse response was detected in this subpopulation. As for NP366$^+$ clonotypes, the distribution could be described by a log-series distribution (Fig. 2*B*), but some samples exhibited significantly more tailiness than expected from a log-series. Values of α were similar in the primary and secondary Vβ7$^+$ response to epitope PA224 (Tables III and IV). Seventy to 156 different Vβ7$^+$ clonotypes per spleen responded to epitope PA224. When corrected for tailiness (overabundance of common sequences), the number of PA224$^+$V7$^+$ CD8 T cells was as high as 250. Combining primary and secondary responses, PA224$^+$Vβ7$^+$ responses were more diverse than either NP366$^+$Vβ8.3$^+$ or NP366$^+$Vβ4$^+$ responses (higher α, $p = 0.0002$; more clonotypes, $p = 0.0001$). However, because 45.1% (±2.4%; $n = 5$) of epitope PA224-specific CD8 T cells express the Vβ7 element, the total number of TCRβ clonotypes responding to PA 224 was not significantly different from the

Table IV. *Estimate of number of epitope-specific CD8$^+$ T cell clonotypes per spleen*

Mouse No.	α (p)[a]	Total No. of Clonotypes per Spleen	Frequency per 10^4 Cells	α (corr.)[b]	Total No. of Clonotypes per Spleen (corr.)	Frequency per 10^4 Cells (corr.)
Epitope NP366						
Vβ8.3 (1°)[c]						
1	2.94 *(0.007)*	33 (12–53)	24	4.07 (0.56)	42 (15–68)	29
2	7.57 (0.15)	79 (43–113)	54			
3	2.46 (0.77)	28 (9–46)	20			
4	1.35 (0.28)	16 (2–30)	12			
Vβ8.3 (2°)						
5	3.9 *(0.003)*	50 (25–74)	31	7.86 (0.41)	84 (39–127)	46
6	3.19 (0.48)	41 (19–63)	26			
7	6.77 *(0.019)*	83 (49–115)	49	7.52 (0.21)	90 (52–125)	52
8	3.37 *(0.016)*	44 (17–69)	27	4.36 (0.52)	53 (20–84)	32
9	2.62 (0.99)	35 (14–54)	22			
10	2.02 *(0.0001)*	27 (9–45)	17	2.82 (0.97)	35 (10–57)	21
11	2.03 *(0.020)*	27 (6–47)	17	3.54 (0.91)	42 (6–75)	25
Vβ4 (2°)						
5	5.86 *(<0.0001)*	68 (34–100)	44	10.92 (0.13)	119 (57–178)	74
6	3.48 *(0.0038)*	42 (17–66)	28	4.88 (0.85)	55 (20–87)	37
7	4.85 (0.14)	57 (27–85)	37			
Epitope PA224						
Vβ7 (1°)						
15	9.23 (0.40)	92 (51–131)	65			
16	6.83 (0.067)	70 (34–104)	50			
Vβ7 (2°)						
5	7 *(0.0005)*	78 (45–109)	51	8.42 (0.56)	89 (52–125)	61
6	9.28 *(0.049)*	101 (63–137)	65	10.9 (0.098)	113 (70–154)	71
17	15.07 *(0.0048)*	156 (97–213)	98	27.0 (0.18)	251 (154–344)	147
18	14.86 (0.15)	154 (94–211)	97			
19	12.87 *(0.0064)*	136 (79–189)	86	17.77 (0.082)	177 (102–248)	114
Vβ8.1/2 (1°)						
20	4.36 *(0.029)*	42.4 (16–67)	34	—[d]		
21	6.29 (0.89)	58.9 (22–93)	46			
22	3.65 (0.87)	36.1 (12–58)	29			

[a] Value of p for fit to log-series distribution, via Monte Carlo test of tailiness (see *Materials and Methods*). Significant ($p < 0.05$) deviations from log-series are italicized.
[b] Corrected (corr.) for tailiness as described in *Materials and Methods* and *Results.*
[c] Vβ element for primary (1°) or secondary (2°) response.
[d] Correction of α estimate was not possible, because, for this sample, dropping the overfrequent clones did not achieve consistency with the log-series distribution.

total response to NP366 (confidence intervals for clonotypes in the spleen overlap, with 150–550 for PA224 and 50–300 for NP366). Because the confidence intervals are fairly wide, we cannot rule out a true diversity difference, but such a difference is likely to be modest if it exists. Of interest, such a difference in precursor frequencies could help explain the observation that PA224-specific CD8 T cells are detected before NP366$^+$ CD8 T cells in influenza-infected mice (17).

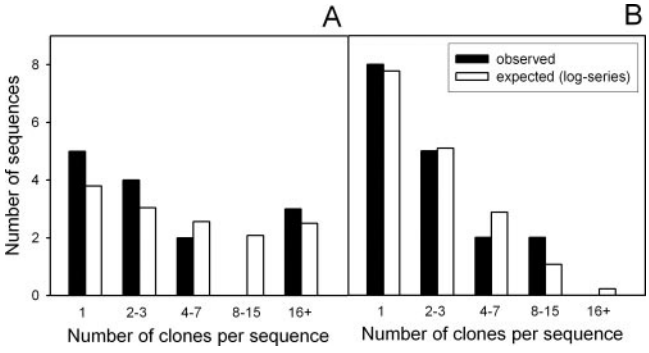

FIGURE 2. Expected and observed patterns of T cell clonotype diversity for representative mice. The observed frequency of NP366$^+$Vβ8.3$^+$ (*A*) and PA224$^+$Vβ7$^+$ (*B*) T cell clonotypes is shown. Expected frequencies were calculated using α estimated from observed frequencies (Table IV). Diversity (estimated by log-series α) is higher for the sample in *B* (note more rare sequences). The sample in *A* exhibited significantly excess tailiness and is typical of samples for which we calculated a corrected α. The sample in *B* did not differ significantly from a log-series distribution.

The values of α were lower for the PA224$^+$Vβ8.1/2$^+$ subpopulation and translated to 35–60 different clonotypes expressing this Vβ element per spleen. Because 10.9% ($\pm$1.6%; $n = 3$) of all PA224$^+$ CD8 T cells express the Vβ8.1/2 element, this extrapolates to 350–600 different PA224$^+$ TCRβ clonotypes in the spleen, consistent with the numbers derived from the Vβ7$^+$ population.

Discussion

Herein, we show that the domination of the epitope NP366-specific T cell response by a few CDR3β sequences present in most mice is not limited to CD8 T cells expressing the Vβ8.3 element, but is exhibited by NP366$^+$ cells expressing a second Vβ element. The MHC class I/peptide NP366 complex is unusual in that its structure is flat, with no prominent structural features (32). This flatness correlates with a NP366$^+$Vβ8.3$^+$ CD8 T cell response that is dominated by a few clonotypes that are reproducibly found in most infected mice. Our results show that the NP366$^+$Vβ4$^+$ subpopulation is also dominated by a few public sequences that are highly expressed in each animal. However, both the NP366$^+$Vβ8.3$^+$ and NP366$^+$Vβ4$^+$ populations include additional T cell clonotypes,

most of which are present at low frequency. Consequently, the overall response to this epitope exhibited substantial diversity. In HLA-A-2$^+$ humans, a public CD8 T cell response to influenza matrix epitope M58 (HLA-A2-restricted) is detected. This MHC class I/peptide complex also lacks prominent features and is dominated by clonotypes expressing a common motif (33). However, even with this restricted CDR3β sequence usage, the CD8 T cell response to this epitope is very diverse (6, 7), consistent with our results.

Our results confirmed previous conclusions that the PA224$^+$Vβ7$^+$ response, unlike the NP366$^+$Vβ8.3$^+$ response, was characterized by private TCR usage (19) and, additionally, showed that the PA224$^+$ CD8 T cell response was polyclonotypic. Approximately 16–90 different Vβ8.3$^+$ clonotypes recognized epitope NP366. The PA224$^+$Vβ7$^+$ response was more diverse, with 70–250 different clonotypes responding to the epitope in each mouse. The total number of responding clonotypes for both epitopes was in the range of 50–550 per spleen. These numbers are less than the number of splenic CD8 T cell clonotypes that responded to MHV epitope S510 or LCMV epitope gp33 by a factor of 2–3 (5), although they are still substantially greater than the diversity determined in studies of other epitopes (1–3, 34).

In these other studies, the precursor frequency to single epitopes was estimated to be 100–200 cells, comprised of 20 different clonotypes. In some instances, extensive sequencing of cDNA clones containing CDR3 from epitope-specific T cells was performed (1, 3). The authors assumed that all epitope-specific clonotypes in the spleen were sequenced and did not perform any extrapolation to determine the total number of epitope-specific CD8 T cells. Because several singletons were identified in these studies, it is likely that additional clonotypes would be detected with further sequencing (1, 3). In another approach, precursor frequencies were determined after adoptive transfer of epitope-specific T cells (2, 34). This approach assumed that equal numbers of transferred and endogenous T cells after Ag exposure equated with equal numbers of precursor cells. However, unlike the transferred cells, the distribution of endogenous cells was nonlinear, so these studies are likely to underestimate the number of clonotypes.

Although our studies and the studies of others suggest that >20 different CD8 T cell clonotypes respond to each epitope (5–7), the precise number of clonotypes responding to each epitope must be considered tentative. The log-series distribution, used in our studies, is often used to describe samples with many rare and a few abundant species. An important assumption of our calculations is that our data can be extrapolated to clonotypes present at very low frequency—clonotypes that were, therefore, not actually sampled in our experiments. Validating these assumptions experimentally would entail sequencing more cDNA clones than is feasible. In this report, we applied a more stringent analysis than used previously to assessing the fit of data to the log-series distribution, and we observed that only a fraction of the NP366$^+$ and PA224$^+$ responses were well described by an unmodified log-series, with lack of fit due primarily to overrepresentation of one to three superabundant sequences. However, none of our major conclusions is likely to be sensitive to this lack of fit, for two reasons. First, correcting for the lack of fit (by decomposing the distribution into a superabundant component and a log-series component) only increases our estimates of diversity; therefore, our inference that CD8 T cell responses are very diverse is robust to the correction. Second, the lack-of-fit correction in most cases increased our estimates of total clonotype diversity only slightly; that is, our tests for tailiness are powerful enough to be detecting mostly subtle deviations from the log-series. A separate source of uncertainty in our estimates of numbers of epitope-specific clonotypes is that they consider sequence diversity only in the TCR β-chain. Including contributions of the TCR α-chain to diversity would further increase our estimates of response diversity.

An important question is how does the level of TCR diversity within a response impact on outcome after viral infection. H2-K^{bm8} mice are more resistant to HSV-1 infection than coisogenic B6 mice. The only difference between H2-K^{bm8} and B6 mice is at the H2K^b allele. Interestingly, the resistance to HSV infection observed in the H2-K^{bm8} mice correlated with the generation of an Ag-specific CTL population that exhibited greater TCR diversity and avidity (35). Given that higher TCR avidity is associated with increased functional capacity (36), the selection of a more diverse repertoire resulted in a more robust HSV-specific CTL response capable of controlling HSV infection.

Public sequences should, in the evolutionary long term, carry less benefit to the animal than private ones. This is because presumably the pathogen population is more likely to evolve to avoid recognition by public sequences. TCR diversity decreases the possibility that mutated pathogens escape CD8 T cell recognition and control (9, 37). Therefore, this limits the ability of persistent viruses, such as HIV and CMV, to escape CTL immunity. A TCR repertoire more limited in diversity lack the flexibility to cope with differences in CTL peptide variants increasing the likelihood of CTL immune escape (37).

Although the immune response to epitopes NP366 and PA224 within individual animals are dominated by a few sequences, the potential importance of rare clonotypes was illustrated in several recent studies of heterologous immunity (12, 38). Cross-reactive T cells may be protective but may also contribute to immunopathology (8). In one example, severe hepatitis induced by hepatitis C virus in two patients was associated with an immune response focused on a single epitope (38). Remarkably, this epitope cross-reacted with an influenza neuraminidase epitope suggesting that prior infection with a common infection contributed to the development of a narrow, immunopathogenic immune response after hepatitis C virus infection. Our results show that even responses that appear to be oligoclonal are diverse. This high clonotypic diversity raises the possibility that most epitopes have the potential to elicit cross-reactive immune responses, perhaps even to several additional epitopes. Mammalian responses to immune challenge may be more complex than previously suspected, both with respect to response to a single challenge and with respect to interplay between responses to different challenges.

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Disclosures

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