

# Conserved T cell receptor usage in primary and recall responses to an immunodominant influenza virus nucleoprotein epitope

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The CD8<sup>+</sup> T cell response to the immunodominant D<sup>b</sup>NP<sub>366</sub> epitope has been analyzed sequentially to determine the prevalence and persistence of different T cell antigen receptor (TCR)Vβ8.3 clonotypes after primary and secondary influenza virus challenge. Based on the length and amino acid sequences of the complementarity-determining region 3 of TCRβ (CDR3β) loop and associated Jβ usage, the same dominant TCRβ signatures were found in the blood, the spleen, and the site of virus-induced pathology in the infected respiratory tract. Longitudinal analysis demonstrated that TCRβ prominent in the antigen-driven phase of response persisted into memory and were again expanded after secondary challenge. A proportion of these high-frequency TCRβ expressed "public" CDR3β sequences that were detected in every mouse sampled, whereas others were found more than once but were not invariably present. Analysis of N-region nucleotide diversity established that as many as 10 different nucleic acid sequences (maximum of four "nucleotypes" in any one mouse) could encode a single public TCRβ amino acid sequence. Conversely, whereas some of the unique, "private" TCRβ achieved a substantial clone size, they were always specified by a single nucleotide. Although there is a strong stochastic element in this response, the public TCRβ seem to represent a "best fit" for this immunodominant epitope, are selected preferentially from the naive TCR repertoire, and assume even greater prominence after secondary challenge.

CD8<sup>+</sup> T cells | T cell receptor repertoire | influenza A virus

Epitope-determined T cell-receptor (TCR) repertoires are selected from a pool of naive precursors estimated to range in size from  $\approx 10^7$  and  $\approx 10^8$  distinct TCRαβ elements (1, 2). Antigen specific CD8<sup>+</sup> T cell responses are sometimes biased toward usage of a particular TCRVα (3–5), but are more commonly determined by the TCRVβ profile (5–15). Most of the specificity of the TCRβ interaction is associated with the third complementarity-determining region (CDR3β) loop, which is generally positioned over the center of the antigenic peptide bound inside the groove of the MHC class I molecule (16, 17). Defining patterns of TCR CDR3β usage, either by determining amino acid sequences or by the spectratyping approach that measures CDR3β length and Jβ usage (18–20), provides a mechanism for following defined T cell populations through the course of an immune response into long-term memory (15, 21, 22).

Longitudinal single-cell analysis of CDR3β profiles in the CD8<sup>+</sup>Vβ7<sup>+</sup> response to an influenza virus acid polymerase peptide (PA<sub>224–233</sub>) presented by the H-2D<sup>b</sup> MHC class I glycoprotein (D<sup>b</sup>PA<sub>224</sub>) provided evidence of substantial diversity in the absence of a single "public" TCRβ signature that could be detected in every mouse (15). A much earlier and more limited study with hybridoma cell lines identified what looked to be a more public response by CD8<sup>+</sup>Vβ8.3<sup>+</sup> T cells specific for the D<sup>b</sup>NP<sub>366</sub> influenza virus nucleoprotein (NP<sub>366–374</sub>) epitope (9). There was no consistent pattern of TCRVα usage by these CD8<sup>+</sup>Vβ8.3<sup>+</sup> T cell hybridomas. This

work explores this CD8<sup>+</sup>Vβ8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> repertoire further by using contemporary tetramer staining, single-cell sorting and PCR-based technology (15). The results provide a stark contrast in clonal diversity for these CD8<sup>+</sup>Vβ7<sup>+</sup>D<sup>b</sup>PA<sub>224</sub><sup>+</sup> and CD8<sup>+</sup>Vβ8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> T cell populations that expand equally and give rise to comparably sized memory T cell populations after primary influenza infection but differ greatly in numbers (CD8<sup>+</sup>Vβ8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup>  $\geq 10 \times$  CD8<sup>+</sup>Vβ7<sup>+</sup>D<sup>b</sup>PA<sub>224</sub><sup>+</sup>) subsequent to secondary virus challenge (23–25).

## Materials and Methods

**Mice and Viral Infection.** Female C57BL/6J (B6, H2<sup>b</sup>) mice were bred at the University of Melbourne. Some were anaesthetized at 6 weeks of age by isoflurane inhalation and infected intranasally (i.n.) with  $10^4$  plaque forming units (pfu) of the HKx31 (H3N2) influenza A virus (26) in 30  $\mu$ l of PBS. Memory mice for secondary challenge experiments were injected i.p. at least 6 weeks previously with  $1.5 \times 10^7$  pfu of the PR8 (H1N1) influenza A virus (27, 28). Both virus stocks were grown in the allantoic cavity of 10-d embryonated hen's eggs and stored in aliquots at  $-80^\circ\text{C}$ . Virus titers were determined as pfu on monolayers of Madin–Darby canine kidney cells.

**Tissue Sampling and Cell Preparation.** Mice were anaesthetized i.p. with 3 mg of ketamine and 0.6 mg of Xylazil (Parnell Laboratories, Alexandria, Australia) and exsanguinated from the axillary artery; total body perfusion was performed with PBS/heparin. Lymphocytes recovered by bronchoalveolar lavage (BAL) of the infected lung were incubated on plastic Petri dishes for 1 h at 37°C to remove macrophages (27). Spleens were disrupted and enriched for CD8<sup>+</sup> T cells by using goat anti-mouse IgG and IgM antibodies (Jackson ImmunoResearch). The longitudinal analysis of TCR repertoire used mice bled by means of the retroorbital sinus. The blood (50–100  $\mu$ l) was collected into 50  $\mu$ l of 10 units/ml heparin in PBS, followed by lysis of the RBCs with 10 ml of ammonium Tris chloride buffer at 37°C for 5 min (25).

**Tetramer and TCRVβ Staining of CD8<sup>+</sup> T Cells.** The D<sup>b</sup>NP<sub>366</sub>-specific CD8<sup>+</sup> T cells were identified by using tetrameric complexes (28) of the influenza virus H-2D<sup>b</sup> MHC class I glycoprotein and the NP<sub>366–374</sub> (ASNENMETM) peptide (29) conjugated to streptavidin-phycoerythrin (Molecular Probes). Lymphocytes were stained with the D<sup>b</sup>NP<sub>366</sub>-phycoerythrin tetramer for 60 min at room temperature, followed by two washes in sort buffer (0.1% BSA in PBS), then anti-CD8α-allophycocyanin (for sorting) or

Abbreviations: TCR, T cell receptor; CDR3β, complementarity-determining region 3 of the TCRβ; D<sup>b</sup>NP<sub>366</sub>, epitope comprised of the nucleoprotein NP<sub>366–374</sub> peptide bound to MHC class I glycoprotein H-2D<sup>b</sup>; D<sup>b</sup>PA<sub>224</sub>, complex of H-2D<sup>b</sup> and virus acid polymerase peptide PA<sub>224–233</sub>; i.n., intranasally; BAL, bronchoalveolar lavage.

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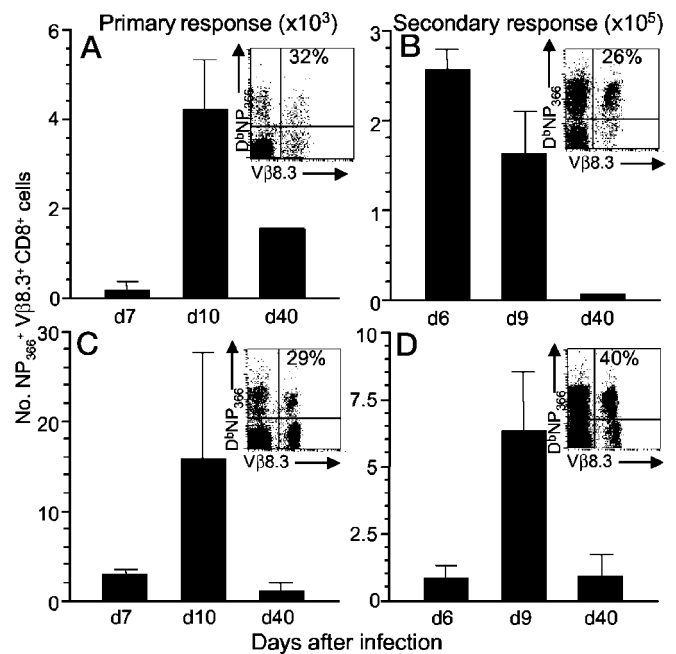
anti-CD8 $\alpha$ -PerCP Cy5.5 (for phenotyping) and anti-V $\beta$ 8.3-FITC (Pharmingen) for 30 min on ice, followed by two further washes. The stained lymphocytes were resuspended in sort buffer ( $2 \times 10^7$  cells per ml) and transferred to polypropylene tubes (BD Biosciences). Spleen or peripheral blood T cells from naive, uninfected mice were stained with anti-CD8 $\alpha$ -phycoerythrin and anti-V $\beta$ 8.3-FITC.

**Isolation of Single CD8 $^+$  T Cells, RT-PCR, and Sequencing.** Lymphocytes were isolated with a MoFlo sorter (Cytomation, Fort Collins, CO) fitted with a Cyclone single-cell deposition unit (15). Single immune (CD8 $^+$ V $\beta$ 8.3 $^+$ D $^b$ NP $_{366}$  $^+$ ) or naive CD8 $^+$ V $\beta$ 8.3 $^+$  T cells were sorted directly into a 96-well PCR plate (Eppendorf) containing 5  $\mu$ l of cDNA reaction mix. Negative controls were interspersed between the samples (1 in 10), and 50–80 cells were sorted per plate. The cDNA mix contained 0.25  $\mu$ l of Sensiscript reverse transcriptase, 1 $\times$  cDNA buffer, 0.5 mM dNTP (all from Qiagen), 0.125  $\mu$ g of oligo dT $_{(15)}$  (Promega), 100  $\mu$ g/ml gelatin (Roche), 100  $\mu$ g/ml tRNA (Roche), 20 units of RNasin (Invitrogen), and 0.1% Triton X-100 (Sigma). After sorting, plates were incubated at 37°C for 90 min for cDNA synthesis, followed by 5 min at 95°C to stop reverse transcriptase activity, and stored at  $-80^\circ\text{C}$ . The V $\beta$ 8.3 $^+$  transcripts were amplified by nested PCR by using 2  $\mu$ l of cDNA for a 25- $\mu$ l amplification reaction. The first-round PCR was performed with 1.5 units of *Taq* polymerase (Invitrogen)/1.5 mM MgCl $_2$ /0.2 mM dNTP (Invitrogen)/10 pmol each of the external sense primer V $\beta$ 8.3-5' (5'-AGCCCTAGAAACAAGGTGAC-3') and the external antisense primer C $\beta$ a (5'-CCAGAAGGTAGCAGAGACCC-3'). A 2- $\mu$ l aliquot of the first-round PCR was used as a template for the nested PCR with the internal sense V $\beta$ 8.3 primer (5'-ACCAGAACAACGCAAGAAGAC-3') and the antisense primer C $\beta$ b (5'-CTTGGGTGGAGTCACATTTCTC-3'). The PCR conditions were 95°C for 5 min followed by 33 cycles of 95°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min followed by 1 cycle of 95°C for 1 min, 57°C for 1 min, and 72°C for 7 min. The PCR products were resolved on a 2% agarose gel, purified with the MiniElute PCR purification kit (Qiagen), and sequenced with 20 pmol of the V $\beta$ 8.3 sense primer by using Big Dye 3.1 (Applied Biosystems). Sequencing products were purified by using Dye Ex 2.1 columns (Qiagen) and sequenced on an Applied Biosystems Prism 3700 sequence analyzer. The V $\beta$ 7 $^+$  transcripts were amplified and sequenced as described in ref. 15.

## Results

The basic aim of these experiments is to define the nature of an antigen-specific CD8 $^+$  T cell repertoire, both within and between individuals responding to a primary or secondary virus challenge and in persistent immune memory (28, 30). The B6 mice were either immunologically naive (primary response) or had been infected i.p. with the PR8 (H1N1) virus at least 8 weeks before i.n. challenge with the HKx31 (H3N2) influenza A virus (secondary response). The PR8 and HKx31 viruses share the same internal components, including the viral nucleoprotein and viral acid polymerase, but the surface influenza virus hemagglutinin and viral neuraminidase glycoproteins are different, and there is no confounding effect of crossneutralization in these prime/boost studies (28, 30).

**Quantification of the V $\beta$ 8.3 $^+$ CD8 $^+$ D $^b$ NP $_{366}$ -Specific T Cell Response.** Typical flow cytometry profiles for CD8 $\alpha^+$  T cells stained simultaneously with a mAb to V $\beta$ 8.3 and the D $^b$ NP $_{366}$  tetramer are shown for BAL (Fig. 1 *A* and *B*) and spleen (Fig. 1 *C* and *D*) populations recovered following i.n. challenge with the HKx31 virus. The B6 mice were either immunologically naive (primary response, Fig. 1 *A* and *C*), or had been infected i.p. at least 8 weeks previously with the PR8 virus (secondary response, Fig.



**Fig. 1.** Kinetics of primary and secondary CD8 $^+$  V $\beta$ 8.3 $^+$ D $^b$ NP $_{366}$  $^+$  responses. Naive or PR8-primed B6 mice were infected i.n. with the HKx31 virus. Lymphocytes were isolated from B6 mice at 7, 10, or 40 d of the primary response, or at 6, 9, or 40 d after secondary challenge. Enriched CD8 $^+$  T cells were stained with the D $^b$ NP $_{366}$ -phycoerythrin tetramer, anti-V $\beta$ 8.3-FITC, and anti-CD8-PerCP Cy5.5 mAbs and analyzed by flow cytometry. The fluorescence-activated cell sorter panels shown on the figures are for day 10 primary (*A* and *C*) and day 9 secondary (*B* and *D*) responses. The cell counts shown here were calculated from the percentage of cells staining and the numbers recovered in the BAL and spleen populations.

1 *B* and *D*). The range of  $\approx 25$ –50% prevalence for the V $\beta$ 8.3 $^+$  component within these CD8 $^+$ D $^b$ NP $_{366}$  $^+$  sets (Fig. 1) is in accord with previous findings (9).

Earlier studies showed that the D $^b$ NP $_{366}$ -specific response dominates influenza virus-specific CD8 $^+$  T cell numbers after secondary (but not primary) exposure of B6 mice to the HKx31 virus (23, 25). The expansion of the V $\beta$ 8.3 $^+$  subset within the CD8 $^+$  D $^b$ NP $_{366}$  $^+$  population parallels the expected profile after secondary challenge (Fig. 1 *B* and *D*). The recall CD8 $^+$  V $\beta$ 8.3 $^+$ D $^b$ NP $_{366}$  $^+$  response in the BAL (Fig. 1*B*) peaked earlier at a level that was more than 100-fold higher than that found after primary infection (Fig. 1*A*). The differential between the primary and secondary CD8 $^+$  V $\beta$ 8.3 $^+$ D $^b$ NP $_{366}$  $^+$  response for maximum counts in the spleen was  $\approx 30\times$ . The CD8 $^+$  V $\beta$ 8.3 $^+$ D $^b$ NP $_{366}$  $^+$  T cell numbers in the spleen then progressively declined to day 40 to give greatly diminished memory T cell population (Fig. 1 *C* and *D*).

Thus, the overall pattern is of a very dynamic response with dramatic changes in virus-specific CD8 $^+$ V $\beta$ 8.3 $^+$ D $^b$ NP $_{366}$  $^+$  T cell numbers from the effector phase to the memory phase (Fig. 1). Furthermore, both the magnitude and kinetics of T cell invasion into the virus-infected lung are greatly enhanced after secondary challenge (Fig. 1*B*). These dramatic changes reflect successive cycles of massive clonal expansion and editing (25). How stable is the profile of TCR repertoire usage throughout the course of this highly dynamic process?

**Primary CD8 $^+$ V $\beta$ 8.3 $^+$ D $^b$ NP $_{366}$  $^+$  TCR $\beta$  Profiles Between Individual Mice.** Immune CD8 $^+$ V $\beta$ 8.3 $^+$ D $^b$ NP $_{366}$  $^+$  T cells recovered from the BAL and spleen of naive B6 mice infected i.n. with the HKx31 virus were sorted to give one cell per well and the CDR3 $\beta$  mRNA was expanded by RT-PCR with  $\approx 80\%$  efficiency to give

**Table 1. Acute phase and memory CDR3 $\beta$  repertoires for different individuals**

CDR3 $\beta$ region	J $\beta$	Length, aa	TCR $\beta$ prevalence, %							
			Acute (day 8)						Memory (day 200+)	
			M1*		M2*		M3*		M4*	M5*
			Spl	BAL	Spl	BAL	Spl	BAL	Spl	Spl
SGGGNTGQL	2.2	9	61	27	31	64	89	50	23.0	21.2
SGGANTGQL	2.2	9	7	36	54	21	6	44	4.1	1.5
RGGANTGQL	2.2	9	—	3	—	5	—	33	1.4	1.5
RGGSTGQL	2.2	9	—	—	2	2	—	—	51.4	3.0
SGGSNTGQL	2.2	9	—	—	3	—	—	—	—	57.6
SGGARTGQL	2.2	9	—	—	—	—	—	—	20.3	—
KGGSNTGQL	2.2	9	13	27	—	—	—	—	—	—
RGGGRTGQL	2.2	9	3	—	6	—	—	—	—	—
RGGGNTGQL	2.2	9	—	—	—	—	—	—	—	1.5
KGGQNTGQL	2.2	9	2	—	—	—	—	—	—	—
SGGGKHRQL	2.2	9	2	—	—	—	—	—	—	—
GGGANTGQL	2.2	9	—	3	—	—	—	—	—	—
GGGENTGQL	2.2	9	—	—	2	—	—	—	—	—
RGGNTGQL	2.2	8	—	—	2	—	—	—	—	—
SGGNTGQL	2.2	8	2	—	—	—	—	—	—	—
SARTANTEV	1.1	9	10	—	—	—	—	—	—	—
SARTGNTEV	1.1	9	2	—	—	—	—	—	—	—
RGAATTEV	1.1	8	—	—	—	2	—	—	—	—
SVAATTEV	1.1	8	—	—	—	2	—	—	—	—
SDWQGRGNTL	1.3	10	—	—	—	—	—	—	—	13.6
SARDGNYAEQ	2.1	10	—	—	—	—	—	3	—	—
SDLAGNAEQ	2.1	9	—	3	—	—	—	—	—	—
RDRESAETL	2.3	9	—	—	—	2	—	—	—	—
SDWGRDEQ	2.6	8	—	—	—	—	6	—	—	—
Total NP <sub>366</sub> specific sequences			61	33	61	42	18	34	74	66

Lymphocytes were obtained from either from BAL or spleens (Spl) of individual B6 mice at 8 d (or from spleens at 200 and 240 d) after i.n. infection with the HKx31 virus, then processed for single cell CDR3 $\beta$  analysis as described in *Material and Methods*. NP, viral nucleoprotein.

\*Individual mice tested.

the product used for sequencing. The V $\beta$ 8.3 CDR3 $\beta$  lengths were designated (31) from the analysis of amino acid sequences, whereas J $\beta$ -element usage was determined after comparison with known genomic sequences (32, 33). The TCR $\beta$  identity was thus based on J $\beta$  usage and the length and amino acid sequence of the CDR3 $\beta$  loop (15).

Two dominant TCR $\beta$  were present in the BAL and spleen of all three mice (Table 1, M1 to M3, first two lines) that were sampled on day 8 of the primary CD8<sup>+</sup>D<sup>b</sup>NP<sub>366</sub>-specific response. Furthermore, although they were less prominent, these same CDR3 $\beta$  signatures were found at significant prevalence in memory spleen populations recovered from different individuals infected more than 200 days previously (Table 1, M4 and M5). Such high-frequency TCR $\beta$  thus represent public amino acid sequences that are selected repeatedly in the CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> T cell response. Furthermore, the same pattern of J $\beta$ 2.2 usage and 9-aa CDR3 $\beta$  length was found for 13 other TCR $\beta$  recovered from these five mice, with 5 of 13 being detected in more than one individual (Table 1). In all, a total of 24 TCR $\beta$  were detected ( $n = 389$  sequences). The amino acid at position 1 was S, K, G, or R for both the public and “private” TCR $\beta$ , and the CDR3 $\beta$  length ranged from 8 to 10 aa (Table 1).

**Sequential Analysis Within Individuals.** A further five mice were primed i.p. with the PR8 (H1N1) virus, then exposed i.n. to the HKx31 (H3N2) virus at least 90 d later. The peripheral blood lymphocyte compartment was sampled acutely on day 8 after the initial PR8 infection and again (day 30) in the early stage of

memory (primary response). All mice were then exsanguinated on day 8 (days 98–148) after the HKx31 challenge and peripheral blood lymphocyte, spleen, and BAL were analyzed (secondary response). The results for one individual are shown in Table 2. A total of 31 different TCR $\beta$  were found in the 1,053 sequences that were analyzed, with 22 of these TCR $\beta$  being unique to individual mice (only in one of five mice). Two of the prominent TCR $\beta$  (SGGGNTGQL and SGGANTGQL; Table 2) were present in every individual (Table 3) sampled in this series and the previous one (Table 1).

The public TCR $\beta$  found at high frequencies (Table 3) in all of the mice tested during the primary or recall response were characterized by the usage of J $\beta$ 2.2, a 9-aa CDR3 $\beta$  loop, and the N-D $\beta$ -N region SGGG or SGGA (Table 3). The remaining five sequences that were recovered from at least 3 of 10 mice (Table 3) also displayed the J $\beta$ 2.2, 9-aa profile. One “consensus” TCR $\beta$  (SGGANTGQL) that was invariably selected in the CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> response (Table 3) was also found once in CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup> T cells recovered from naïve, uninfected mice (total of 361 sequences analyzed; data not shown). Clearly, many more naïve TCR $\beta$  signatures would need to be examined before any more precise definition of the preimmune TCR $\beta$  repertoire can be made.

The 9 of 31 TCR $\beta$  that were found in more than one of the five sequentially sampled mice dominated the CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> response. These “repeat” CDR3 $\beta$  profiles were expressed on 80.4%  $\pm$  20.1% of the CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> T cells analyzed on day 8, 76.6%  $\pm$  22.8% on day 30, and 93.4%  $\pm$  9.0% on day 148.

**Table 2. Sequential CDR3 $\beta$  analysis within an individual mouse**

CDR3 $\beta$ region*	J $\beta$	Length, aa	TCR $\beta$ prevalence, %				
			Blood		Spleen		BAL
			day 8 (1°)	day 30 (1°)	day 138 (2°)	day 138 (2°)	day 138 (2°)
SGGSNTGQL	2.2	9	54	22	56	29	65
SGGGNTGQL	2.2	9	18	22	13	6	6
SGGANTGQL	2.2	9	14	22	13	41	23
KGGGNTGQL	2.2	9	9	—	6	6	—
KGGSNTGQL	2.2	9	—	—	—	3	—
RGGGNTGQL	2.2	9	—	—	6	12	—
RGGGNTGQL	2.2	9	5	—	—	3	—
RGGANTGQL	2.2	9	—	7	—	—	—
KAGGNTGQL	2.2	9	—	—	4	—	—
GGGNTGQL	2.2	9	—	—	2	—	—
SGGARTGQL	2.2	9	—	—	—	—	6
SDRGRDTGQL	2.5	10	—	7	—	—	—
SDGTDY	1.2	6	—	7	—	—	—
SGGADTGQL	1.3	9	—	7	—	—	—
SDAGGRDEQ	2.6	9	—	7	—	—	—
Total NP <sub>366</sub> specific sequences			22	14	48	34	35

The primary TCR $\beta$ V8.3 repertoire (1°) was analyzed from single CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> cells recovered from peripheral blood on day 8 and day 30 after i.p. challenge with the PR8 virus. The sequences for the secondary TCR $\beta$ V8.3 repertoire (2°) were obtained from peripheral blood, BAL, and spleens on day 8 after subsequent i.n. infection with HKx31 virus (day 130 after the primary PR8 challenge).

\*The data are for mouse M9.

The perception that the recall response narrows to a more consensus profile is reinforced by the fact that, given the limited amount of blood that can be obtained from mice in survival experiments, many fewer T cells were analyzed on day 8 and day 30 (total 137) after the initial PR8 infection than from the peripheral blood lymphocyte, BAL, and spleen (total 916) following the HKx31 challenge.

Despite this >6-fold difference in sample size, some TCR $\beta$  were detected only in the blood following the initial exposure to PR8 and did not reemerge in the recall response. Most (12 of 18) of the TCR $\beta$  sequences, were private and were found in one of five of these sequentially sampled mice and were also absent from the five that were analyzed at a single time point (Table 1). All 12 lacked the characteristic 9-aa length and J $\beta$ 2S2 profile (Tables 1 and 3). A further 4 of 11 TCR $\beta$  detected only after secondary challenge also showed this private profile, but these

could easily have been missed in the more limited analysis of the primary response.

**Nucleotide Diversity Within Particular CDR3 $\beta$  Amino Acid Signatures.**

The variation permissible in the third nucleotide (“wobble”) encoding a particular amino acid in a TCR $\beta$  sequence allows a more precise definition of clonality. Evidence of considerable N-region nucleotide diversity was found within each of the public TCR $\beta$  that were repeated in this series of five mice (Tables 4 and 5). Although it was not necessarily the case that such public TCR $\beta$  were numerically dominant in any individual response (Tables 1, 2, and 4), the extent of this nucleotide diversity was significantly greater ( $P > 0.001$ ) than for those that were present in <5 but >2 mice (Table 5). Furthermore, the private TCR $\beta$  that were found only once were always progeny of a single nucleotide (Table 5).

Looking at all five mice (Table 5), we found a total of seven different nucleotypes encoding SGGANTGQL. Similarly, the overall SGGGNTGQL response was comprised of 10 clonotypes. Furthermore, although the most frequently repeated nucleotide predominated throughout for the sequentially sampled mouse shown in Table 4, this was not necessarily the case for the other individuals analyzed in this way (data not shown). However, all of the 7 SGGANTGQL clones and 5 of 10 SGGGNTGQL clones found after primary PR8 infection were present again in the recall response, establishing that the majority produced progeny T cells that enter the memory pool.

**Discussion**

The present CDR3 $\beta$  analysis of CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> T cells, together with our prior dissection of the CD8<sup>+</sup>V $\beta$ 7.1<sup>+</sup>D<sup>b</sup>PA<sub>224</sub><sup>+</sup> repertoire (15), provide systematic, longitudinal studies of primary and secondary responses to different epitopes within the same virus. The characteristics in common are that the likelihood of persistence into memory reflects the clonal burst size (34) achieved during the antigen-driven acute response and that the clonotypic profile of T cell memory shows substantial stability in the long term (22). Also, although TCR $\beta$  that are

**Table 3. Public and repeated CDR3 $\beta$  sequences that were found in at least three of eight mice**

CDR3 $\beta$ region	J $\beta$	Length, aa	TCR $\beta$ prevalence, %	
			Primary	Secondary
SGGANTGQL	2.2	9	19.1 (8)	38.3 (5)
SGGGNTGQL	2.2	9	36.2 (8)	6.8 (5)
SGGSNTGQL	2.2	9	15.9 (5)	19.3 (5)
RGGGNTGQL	2.2	9	8.5 (5)	1.4 (2)
KGGGNTGQL	2.2	9	1.6 (2)	15.0 (3)
RGGANTGQL	2.2	9	1.6 (7)	—
RGGGNTGQL	2.2	9	0.6 (3)	11.5 (3)
No. of NP <sub>366</sub> specific sequences (mice)			503 (8)	828 (5)

A total of eight mice ( $n = 503$ ) were sampled after HKx31 or PR8 primary infection, and five mice ( $n = 828$ ) were sampled after secondary challenge. Values in parentheses correspond to the number of mice in which a particular sequence was found. Samples of the primary data are shown in Tables 1, 2, and 4.

**Table 4. Nucleotide and amino acid diversity profiles for a sequentially sampled mouse**

CDR3 $\beta$ region*	Nucleotide prevalence, %				
	Blood			Spleen	BAL
	Day 8 (1°)	Day 30 (1°)	Day 148 (2°)	Day 148 (2°)	Day 148 (2°)
<b>Public</b>					
<u>SGGANTGQL</u>	12.1	20.0	27.1	25.6	20.5
AGTGGGGGGGCAAA	(9.1)	(10.0)	(20.3)	(17.2)	(8.7)
AGTGGGGGGGCTAA	(3.0)	(5.0)	(4.3)	(6.9)	(5.9)
AGTGGTGGGGCAAA	—	(5.0)	(2.5)	(1.5)	(5.9)
<u>SGGGNTGQL</u>	18.2	10.0	4.9	3.4	8.2
AGTGGGGGGGGAAA	(9.1)	(10.0)	—	(1.8)	(4.1)
AGTGGGGGAGGAAA	(3.1)	—	(1.6)	(0.8)	(1.6)
AGTGGGGGGGGGAA	—	—	—	—	(0.8)
AGTGGGGGGGGGCAA	(6.0)	—	(3.3)	(0.8)	(1.6)
<b>Repeated</b>					
<u>SGGSNTGQL</u>	21.2	70.0	9.8	7.7	9.0
AGTGGGGGCTCAA	(19.9)	(10.0)	(6.6)	(3.4)	(5.8)
AGTGGGGGGTCAA	(1.3)	(60.0)	(3.2)	(4.3)	(3.2)
<u>RGGGNTGQL</u>	3.0	—	19.7	20.5	14.8
AGAGGAGGGGGGAA	(3.0)	—	(2.5)	(9.4)	(8.2)
AGGGGTGGGGGGAA	—	—	(17.2)	(11.1)	(6.6)
<u>RGGSNNTGQL</u>	3.0	—	0.8	—	3.3
<u>KGGGNTGQL</u>	18.2	—	32.8	32.5	28.7
<u>SDAANTEV</u>	—	—	0.8	1.7	3.3
<b>Private</b>					
<u>SGGGRSQQL</u>	—	—	0.8	0.9	—
<u>SDDRGRDQDTQ</u>	18.2	—	—	0.9	—
<u>SGGGTTGQL</u>	—	—	—	—	0.8
<u>KAGGNTGQL</u>	—	—	3.3	6.8	10.7
<u>KGGANTGQL</u>	—	—	—	—	0.8
<u>SEGAETL</u>	3.0	—	—	—	—
<u>SEQRLGRYEQ</u>	3.0	—	—	—	—

Nucleotide diversity for public CDR3 $\beta$  (Table 2) that were found in all of the mice, repeated CDR3 $\beta$  signatures recovered from at least two different individuals, or unique private CDR3 $\beta$  signatures. Nucleotide sequences are not provided when the TCR $\beta$  represented the progeny of a single clonotype. 1°, primary response; 2°, secondary response.

\*The data are for mouse M10.

prominent after primary infection continue to be detected after secondary challenge, stochastic events (35) clearly contribute to the recall response. More evidence for the emergence of “novel” TCR $\beta$  after secondary antigen stimulation was found for the CD8<sup>+</sup>V $\beta$ 7.1<sup>+</sup>D<sup>b</sup>PA<sub>224</sub><sup>+</sup> than the CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> re-

sponse, although all such analyses probably underestimate the diversity of memory (36) represented by unique, private clonotypes that are present at very low frequencies (15).

Interestingly, although no public TCR $\beta$  (37) were found for the CD8<sup>+</sup>V $\beta$ 7.1<sup>+</sup>D<sup>b</sup>PA<sub>224</sub><sup>+</sup> response (15), such sequences are a prominent feature of the CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> repertoire. These repeat CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> TCR $\beta$  are characterized by the almost exclusive usage of J $\beta$ 2.2 and a 9-aa CDR3 $\beta$ , whereas the CDR3 $\beta$  loop of D<sup>b</sup>PA<sub>224</sub>-specific T cells is generally shorter (6 aa) and has a more diverse J $\beta$  (1.1, 1.4, and 1.6) profile. The likely reasons for these differences rest in the molecular characteristics of the CDR3 $\beta$ -D<sup>b</sup>NP<sub>366</sub> and D<sup>b</sup>PA<sub>224</sub> interactions (5, 14). The structure of D<sup>b</sup>NP<sub>366</sub> is known (38), and the solution of D<sup>b</sup>PA<sub>224</sub> may be imminent (J. Rossjohn, personal communication), but there are as yet no cocrystals with representative TCR for either of these epitopes. We have not attempted to analyze TCR $\alpha$  expression in these experiments, but the spectrum of TCR $\alpha$  usage in the CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> response shows considerable variability (ref. 9 and K.K., unpublished data), and it seems likely that diversity is largely a function of the CDR3 $\beta$  profile.

Although the secondary CD8<sup>+</sup> T cell response to D<sup>b</sup>NP<sub>366</sub> response is much larger than that to D<sup>b</sup>PA<sub>224</sub> (23, 25, 39), an accumulating body of evidence indicates that the TCR-D<sup>b</sup>PA<sub>224</sub> interaction is of higher avidity (40, 41). The correlation of greater diversity and higher avidity suggests the possibility that

**Table 5. Nucleotide diversity in CDR3 $\beta$  amino acid sequences for mice analyzed longitudinally**

Mouse*	Public (five of five mice)	Repeated (less than five and at least two mice)	Private (one mouse)
M10	3–4	1–2	1
M9	2–3	1–2	1
M8	2–3	1–2	1
M7	1–3	1–2	1
M6	1–3	1–2	1
Mean <sup>†</sup> $\pm$ SD	2.5 $\pm$ 0.97	1.4 $\pm$ 0.5	1 $\pm$ 0
Total in five mice	7–10	1–4	1

The extent of nucleotide diversity within public, repeated, and private TCR $\beta$  signatures is shown for five mice that were sampled sequentially through the primary and recall response (Tables 2 and 4).

\*More detailed data for mice M9 and M10 are shown in Tables 2 and 4, respectively. At least one private TCR $\beta$  in these mice accounted for >10% of the total analysed.

<sup>†</sup>The mean values are all significantly different ( $P > 0.001$ ).

the development of an appropriate binding event between a selected TCR CDR3 $\beta$  and the immunogenic epitope is much more readily achieved for D<sup>b</sup>PA<sub>224</sub> than for D<sup>b</sup>NP<sub>366</sub>. The level of diversity may reflect the selection of dominant characteristics important for specificity. The CD8<sup>+</sup> V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> T cells clearly “prefer” to use a limited set of public TCR $\beta$  and draw these from a spectrum of related nucleotide sequences. The use of the same amino acid sequences encoded by different gene rearrangements is evidence for strong antigen selection for these public TCR $\beta$ . This implies that optimal recognition of the D<sup>b</sup>NP<sub>366</sub> complex requires a combination of structural factors which are only found within the specific TCRV $\beta$ 8.3/CDR3 $\beta$ /J $\beta$ 2.2 combination (5, 36). Although large clone sizes can be achieved by unique private CDR3 $\beta$  that bind D<sup>b</sup>NP<sub>366</sub>, these are only ever specified by a single nucleotide sequence and tend to be less prominent after secondary challenge.

There is also the possibility that higher avidity T cells may be preferentially deleted during the resolution phase of the host response. If this possibility were to bias profiles of TCR retention by, for example, selectively diminishing the prevalence of public CD8<sup>+</sup>V $\beta$ 7.1<sup>+</sup>D<sup>b</sup>PA<sub>224</sub><sup>+</sup> TCR (42), we might expect to see obvious changes in repertoire between the initial stage of antigen-driven clonal expansion and the development of established T cell memory. However, no such effects have been found for primary CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> or CD8<sup>+</sup>V $\beta$ 7.1<sup>+</sup>D<sup>b</sup>PA<sub>224</sub><sup>+</sup> responses. In addition, although there is evidence of preferential tumor necrosis factor- $\alpha$ -mediated loss of the CD8<sup>+</sup>V $\beta$ 7.1<sup>+</sup>D<sup>b</sup>PA<sub>224</sub><sup>+</sup> T cells during the acute response, this is limited to the small minority of highly

activated, tumor necrosis factor-receptor positive T cells recovered from the virus-infected lung (41). The TCR $\beta$  profiles are essentially equivalent for both the CD8<sup>+</sup>V $\beta$ 8.3<sup>+</sup>D<sup>b</sup>NP<sub>366</sub><sup>+</sup> and CD8<sup>+</sup>V $\beta$ 7.1<sup>+</sup>D<sup>b</sup>PA<sub>224</sub><sup>+</sup> BAL and spleen populations, indicating that this differential editing process (if active) does not modify the repertoire.

Analysis of antigen-presenting cells recovered directly *ex vivo* from infected mice indicates that, although D<sup>b</sup>NP<sub>366</sub> can be detected on epithelial cells, macrophages, and dendritic cells, D<sup>b</sup>PA<sub>224</sub> is found only on dendritic cells (43). A formal possibility is that the much wider distribution of D<sup>b</sup>NP<sub>366</sub> on suboptimal, “nonprofessional” antigen-presenting cells skews the response to use of the “best fit” (44, 45), public TCR $\beta$ . Studies with viruses modified by reverse genetic technology to change the spectrum of epitope presentation may provide further information. Hopefully, experiments such as these will help to develop a better understanding of the nature of immunodominance (24, 46) in virus-specific CD8<sup>+</sup> T cell responses and the role that TCR repertoire usage and diversity plays in such processes.

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