

Memory TCR repertoires analyzed long-term reflect those selected during the primary response

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Abstract

Normal T cell repertoire selection and evolution in antigen-specific responses is poorly understood. We have recently described an MHC class I-restricted response characterized by an overwhelming expansion of CD8 cells expressing a $V_{\beta}10$ TCR, thus allowing the identification of antigen-selected cells directly *ex vivo*. Our present strategy to follow the overall TCR repertoire selection was to monitor the expression of a particular TCR α chain ($V_{\alpha}8$) on antigen-selected $V_{\beta}10^{+}$ cells by four-color flow cytometry. We demonstrate that while there is substantial variation among the responder mice in $V_{\alpha}8$ usage, the repertoires of individual animals remain relatively stable over long periods of time (>1 year), with or without repeated antigenic challenge. Thus if any evolution of this response occurs upon re-exposure to antigen, it would appear not to skew the TCR repertoire established during the primary response.

Introduction

The repertoire of antigen receptors expressed by CD4 or CD8 T cells during an *in vivo* immune response is selected by antigenic peptides bound to MHC class II or class I molecules respectively. In responses directed towards a single immunodominant epitope, examples of both highly restricted and relatively diverse TCR repertoires have been reported (reviewed in 1). However, it is not apparent whether these features of the repertoire are stable and are expressed at all stages of the response or whether they evolve over time. The long-term persistence of particular clones has sometimes been observed in man (2–5), but it is difficult to extrapolate from these studies to what extent they are representative of the total repertoire in a given response. The assessment of the susceptibility of a given repertoire to *in vivo* selection is often indirect, since low precursor frequencies necessitate the analysis of T cell clones or hybridomas that may have been subject to bias from *in vitro* culture.

To investigate how antigen shapes the repertoire, we have studied the CD8 T cell response to an immunodominant

epitope (CW3 170–179) expressed on P815-CW3 transfected cells (6,7). Certain features of this response enable antigen-selected cells to be easily identified *ex vivo* without further manipulation. After *i.p.* injection of P815-CW3 cells, there is an expansion of K^d-restricted CD8⁺ cytotoxic T lymphocytes (CTL) that express a single V_{β} element ($V_{\beta}10$). At the peak of the response, a substantial expansion of CW3-specific $V_{\beta}10^{+}$ CD8⁺ cells is readily detectable in peripheral blood lymphocytes (PBL), these cells express a phenotype characteristic of activated cells: CD62L⁻, CD45RB⁻, CD44⁺ (8). Having a system in which antigen-selected T cells could be identified *ex vivo* from PBL allowed us to follow the development of the TCR repertoire in individual mice over time. We have developed two independent strategies for longitudinal repertoire analysis. One approach is based on PCR amplification and sequencing of $V_{\beta}10$ TCR from single $V_{\beta}10^{+}$ CD62L⁻CD8 T cells sorted by flow cytometry (9). For simplicity, we used only $V_{\beta}10$ - $J_{\beta}1.2$ primer combinations, since 60% of CW3-specific clones analyzed used the $J_{\beta}1.2$

gene segment (6,10). This first approach demonstrated that the $V_{\beta}10$ - $J_{\beta}1.2$ TCR repertoires selected in individual mice were limited in size and showed relatively little evolution between primary and secondary responses. However, it was still possible that the remainder of the CW3-specific $V_{\beta}10$ TCR repertoire (TCR using J_{β} segments other than $J_{\beta}1.2$) might show a different pattern of selection or evolution during the response. One possibility would be to perform the PCR amplifications with primers specific for the additional 11 J_{β} gene segments. While feasible, this would require the processing of a large number of PCR amplifications, making the analysis of large numbers of animals over time cumbersome. Thus, we also developed the present, simpler strategy of TCR repertoire analysis that permits an assessment of potential fluctuations in the overall $V_{\beta}10$ repertoire by analyzing expression of a particular V_{α} TCR on the surface of antigen selected cells. We had found previously that V_{α} usage in this response was not uniform (6,10). In particular, only ~15% of the expanded $V_{\beta}10^{+}CD8^{+}$ cells from pools of immune mice were labeled with the $V_{\alpha}8$ mAb B21.14 (7). We have now determined that $V_{\alpha}8$ usage by antigen-selected cells is widely variable between individual immune animals and we have thus exploited this feature as an indicator of potential repertoire evolution over long time periods and after multiple immunizations.

Methods

Immunizations

Female DBA/2 mice (Harlan CPB, Zeist, Netherlands) were injected i.p. with viable syngeneic (H-2^d) P815 tumor cells transfected with the HLA-CW3 gene [P815-CW3 (11)] which had been maintained as an ascitic tumor, serially passaged in nude mice. Primary immunizations were at 2–4 months of age and used 2×10^7 P815-CW3 cells, subsequent immunizations at the times indicated were with either 2×10^6 or 2×10^7 cells.

Cell preparations

PBL were purified from freshly collected heparinized blood using Ficoll-Hypaque centrifugation (Pharmacia Biotech AB, Uppsala, Sweden).

Antibodies and flow cytometric analysis

Directly conjugated mAb were used for the four-color flow-cytometric analysis. Phycoerythrin (PE)-conjugated anti- $V_{\alpha}8$ [B21.14 (12)] was purchased from PharMingen (San Diego, CA) and anti-CD8 α (53.6.7) was purchased as a Red613-conjugate from Gibco/BRL (Life Technologies, Gaithersburg MD). Biotinylated anti-CD62L [MEL-14 (13)] and FITC-conjugated anti- $V_{\beta}10$ [B21.5 (12)] were prepared in our laboratory. The biotinylated reagent was revealed with streptavidin-allophycocyanin (Caltag, San Francisco, CA). Samples were analyzed using a FACStar Plus equipped with argon-ion and helium-neon lasers (Becton Dickinson, Mountain View, CA). The cytometer was calibrated by eye using CaliBRITE beads (Becton Dickinson) of 6.6 μm diameter for the first three colors and dark red latex sulfate 4.0 μm diameter beads (Molecular Probes, Eugene, OR) for the fourth color. Analysis was per-

formed using Lysys II software after gating on viable lymphocytes using a combination of forward and side light scatter (linear amplification). The mAb to CD62L gave a bimodal staining pattern of CD8 cells (8), the high and low intensity populations thus defined are hereafter referred to as positive and negative populations. Where values for pooled mice are given in the text, these are calculated as mean percentages \pm SD.

Statistical estimation of repertoire size

We estimated the repertoire size from the mean and SD of the % $V_{\alpha}8^{+}$ cells in gated populations of $V_{\beta}10^{+}CD62L^{-}CD8^{+}$ PBL, as determined by flow cytometry. This approach is based on two assumptions: (i) that all clones of the repertoire are of equal size and (ii) that the observed variation in this gated population is exclusively due to oligoclonal sampling effects. A total of 5000–10,000 gated cells was analyzed for each sample, ensuring that variability in $V_{\alpha}8$ expression was not due to analysis of too few events. For a repertoire size of N and a probability of P for a given V_{α} of being sampled, the binomial standard deviation is given by

$$\sigma = \sqrt{\frac{P(1-P)}{N}}$$

Using this equation, the estimated average repertoire size is obtained by substituting P and σ respectively for the mean and SD of the fractions observed and solving for N .

Results

Variability of $V_{\alpha}8$ usage among antigen-selected cells in the CW3 response

We have previously shown that during the first few months of the CW3 response, elevated proportions (up to ~80%) of $V_{\beta}10^{+}$ T cells are found exclusively in the CD62L $^{-}CD8$ subset, with no enrichment among CD8 cells of the reciprocal CD62L $^{+}$ phenotype (8). Moreover, the CD62L $^{-}CD8$ subset is itself substantially expanded in immune mice and accounts for up to 70% of the CD8 cells, compared with <10% in control mice. Thus, the $V_{\beta}10^{+}CD62L^{-}$ subset represents a CD8 cell population highly enriched for antigen-selected cells. In the present study we use four-color flow cytometry to analyze $V_{\alpha}8$ expression on $V_{\beta}10^{+}$ cells in the CD62L $^{-}CD8$ cells. For the $V_{\beta}10^{+}$ cells included in the CD62L $^{-}CD8$ subset, we observed a large variation between individual immune mice in the proportion of $V_{\alpha}8^{+}$ cells, ranging from <1% to almost 30% (Fig. 1A). In contrast, little variation was observed in $V_{\alpha}8$ usage among $V_{\beta}10^{+}$ cells in the reciprocal CD62L $^{+}$ subset (Fig. 1B) where no expansion of $V_{\beta}10^{+}$ cells was detected (Fig. 1C). Moreover, a relatively narrow range of $V_{\alpha}8$ usage was found among $V_{\beta}10^{+}CD8$ cells from non-immune controls ($6.9 \pm 1.2\%$). The variability in $V_{\alpha}8$ usage appeared not to be related to the slight variations in the magnitude of individual responses as assessed either by the percentage of $V_{\beta}10^{+}$ cells within the expanded CD62L $^{-}$ subset (Fig. 1C) or by the proportion of CD8 $^{+}$ cells that were both $V_{\beta}10^{+}$ and CD62L $^{-}$ (data not shown).

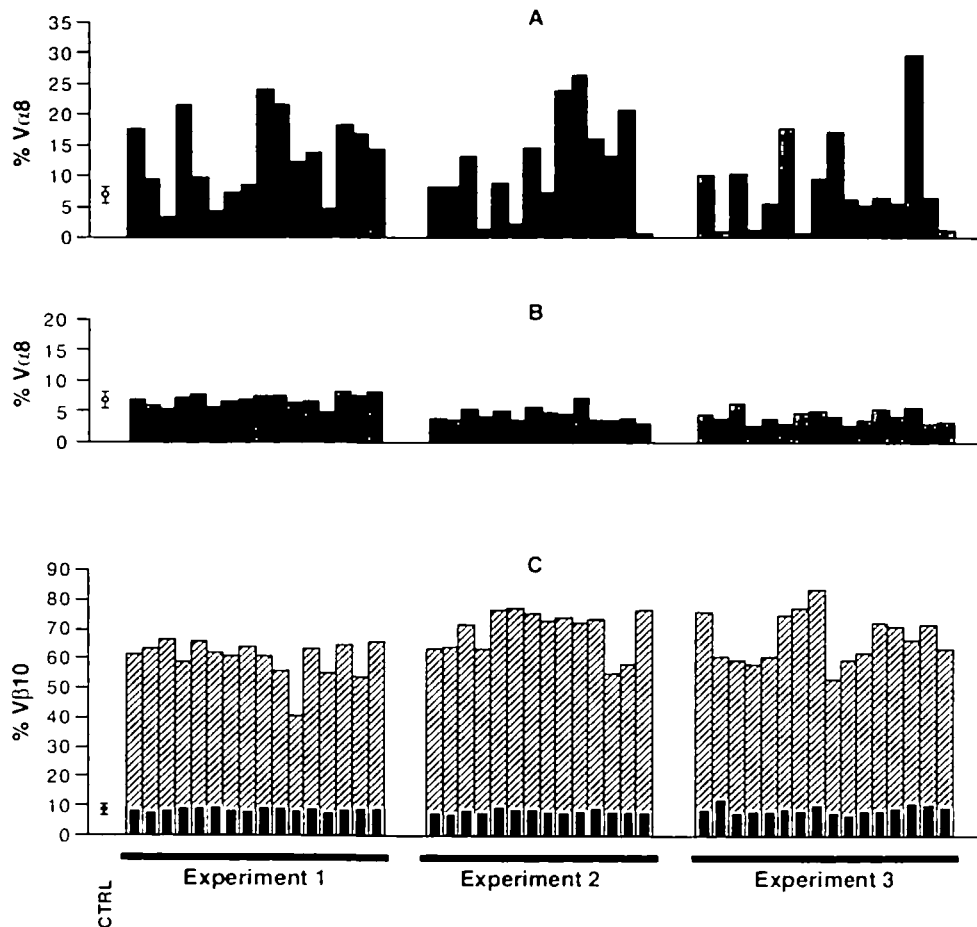


Fig. 1. Antigen-selected cells from individual CW3-immune mice display extremely variable usage of $V_{\alpha}8$. PBL from mice immunized 13–14 days previously with P815-CW3 cells were stained with mAb to CD8, CD62L, $V_{\beta}10$ and $V_{\alpha}8$ and analyzed by four-color flow cytometry. Each histogram bar represents data from an individual mouse and three independent experiments are shown. (A) Percentage of $V_{\alpha}8^{+}$ cells among $V_{\beta}10^{+}CD62L^{-}CD8^{+}$ cells, (B) percentage of $V_{\alpha}8^{+}$ cells among $V_{\beta}10^{+}CD62L^{+}CD8^{+}$ cells, (C) percentage $V_{\beta}10^{+}$ cells among $CD62L^{-}CD8^{+}$ cells (hatched bars) and $CD62L^{+}CD8^{+}$ cells (black bars). PBL from 20 non-immune age-matched control mice (CTRL) were also analyzed and the mean percentages (\pm SD) for $V_{\alpha}8^{+}$ cells among $V_{\beta}10^{+}CD8^{+}$ cells (A and B) and $V_{\beta}10^{+}$ cells among $CD8^{+}$ cells (C) are indicated.

Relative stability of the $V_{\alpha}8$ TCR repertoire during the CW3 response over at least 1 year

It was conceivable that the anti-CW3 repertoire would eventually reach an equilibrium over time that might show less variation among individual animals. Therefore, the same mice were analyzed for $V_{\alpha}8$ usage among antigen-selected cells at different times after immunization with P815-CW3 cells. Late in the primary response, at day 41, $V_{\alpha}8$ usage remained similar to that found at the peak of the response (Table 1). On day 45, seven of 12 mice were boosted (nos 7, 9, 11, 12, 14, 15 and 16), resulting in an increase in the overall level of the response for these mice (Fig. 2). However, little change in $V_{\alpha}8$ usage was observed, whether or not the mice had been boosted (Table 1, days 41–64). Similar results were obtained in two further independent experiments, one of which is analyzed in Fig. 3. As shown in Fig. 3, a significant correlation was found between the proportion of antigen-selected cells expressing $V_{\alpha}8$ at the peak of the primary response and the proportion found either late in the primary

response ($P < 0.00005$) or after a secondary immunization ($P < 0.002$). Remarkably, mice that had elevated $V_{\alpha}8$ levels early in the response continued to display elevated levels ~1 year after primary immunization, even though the proportion of $V_{\beta}10^{+}CD62L^{-}CD8^{+}$ cells at this stage was considerably lower than at the peak of the response (Table 1 and Fig. 2). Moreover, the levels of $V_{\alpha}8$ usage 10 days after a boost given 1 year after primary immunization still reflected those found at the peak (day 14) of the primary response (Table 1). Thus, in long-duration time course experiments during which large fluctuations occurred in the magnitude of the CW3 response (Fig. 2), the proportion of $V_{\alpha}8^{+}$ cells among the antigen-selected cells remained relatively stable (Table 1).

The phenotypic monitoring of $V_{\alpha}8$ TCR expression by antigen-selected cells revealed a subtle feature of the CW3 response. As shown in Fig. 4, some mice select $V_{\alpha}8^{+}V_{\beta}10^{+}$ T cells that can clearly be divided into subpopulations on the basis of the intensity of $V_{\alpha}8$ staining. More than half of the CW3-immune mice in two separate experiments displayed at

Table 1. The variable usage of $V_{\alpha}8$ by antigen-selected cells found between individual mice is stable at different stages of the CW3 response

Mouse no	CD62L subset	Boost at day		% $V_{\alpha}8$ among $V_{\beta}10^+$ CD8 subset at day					
		45	357	14	41	56	64	350	367
7	neg	+	+	10.2	10.9	17.0	13.6	16.4	8.9
9	neg	+	+	1.3	4.3	2.1	1.9	3.9	0.5
11	neg	+	+	1.5	4.6	2.0	1.6	4.5	0.8
12	neg	+	+	5.9	8.4	13.0	10.6	7.4	12.6
14	neg	+	+	17.8	28.1	24.0	27.0	24.5	19.4
15	neg	+	+	1.0	4.1	1.8	1.4	3.9	5.3
16	neg	+	+	9.7	12.5	14.0	12.0	18.0	20.3
18	neg	-	+	17.2	33.9	22.0	23.3	31.2	22.1
19	neg	-	+	6.4	7.5	9.6	8.4	TFC ^a	3.4
21	neg	-	+	5.6	14.0	9.1	5.3	5.8	3.3
22	neg	-	+	23.1	20.8	NT ^b	24.8	33.7	48.1
28	neg	-	+	6.8	12.6	10.0	7.5	7.0	6.5
7	pos	+	+	4.7	5.3	9.8	6.1	9.9	8.2
9	pos	+	+	4.2	3.3	5.1	3.8	3.9	2.7
11	pos	+	+	3.1	6.0	6.4	5.2	6.2	3.6
12	pos	+	+	4.1	5.0	8.4	6.7	5.4	5.3
14	pos	+	+	3.3	10.0	15.0	10.3	14.8	12.9
15	pos	+	+	5.0	7.3	6.2	5.9	4.3	5.2
16	pos	+	+	5.3	1.2	8.3	8.3	16.6	18.1
18	pos	-	+	4.4	6.2	5.8	7.7	17.4	14.0
19	pos	-	+	3.0	4.9	6.7	6.4	4.8	4.6
21	pos	-	+	4.0	3.3	6.1	7.0	6.7	4.1
22	pos	-	+	4.9	TFC	NT	11.1	32.1	29.2
28	pos	-	+	3.4	8.6	6.1	7.1	7.4	6.3

PBL from individual CW3-immune mice from experiment 3 of Fig. 1 (boosted at day 45 and/or day 357 as shown) were isolated at the indicated times after primary immunization, stained with mAb to $V_{\alpha}8$, $V_{\beta}10$, CD62L and CD8 and analyzed by four-color flow cytometry. The percentage of $V_{\alpha}8^+$ cells among $V_{\beta}10^+$ cells gated as CD62L⁻ or CD62L⁺ subsets of CD8 cells is indicated. The proportion of $V_{\beta}10^+$ cells among CD62L⁻CD8 cells at each time point is shown in Fig. 2 for pooled mice from this experiment.

^aToo few cells in gated subset to accurately analyze.

^bNot tested.

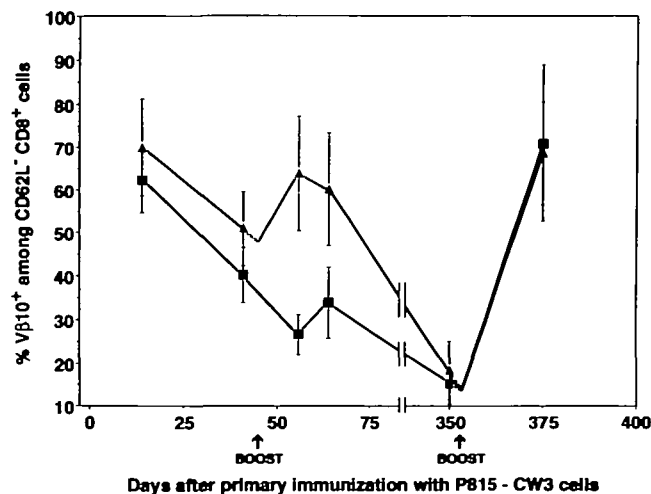


Fig. 2. Changing proportions of antigen-selected $V_{\beta}10^+$ cells over different stages of the response. PBL from CW3-immune mice were stained with mAb to CD8, CD62L, $V_{\beta}10$ and $V_{\alpha}8$ and analyzed by four-color flow cytometry. The mean percentage (\pm SD) of $V_{\beta}10^+$ cells among CD62L⁻CD8 cells is shown for groups of mice boosted at days 45 and 357 (triangles) or at day 357 only (squares). The analysis of $V_{\alpha}8$ expression from the same samples is shown in Table 1.

least two levels of $V_{\alpha}8$ staining on antigen-selected cells (data not shown). The two discernible $V_{\alpha}8^+V_{\beta}10^+$ subpopulations show similar levels of $V_{\beta}10$ staining but vary in $V_{\alpha}8$ intensity. We consider the most likely explanation for the $V_{\alpha}8$ dim population is that these cells express a second cell-surface α chain that competes with $V_{\alpha}8$ for pairing with $V_{\beta}10$, thus reducing the total $V_{\alpha}8$ available for antibody labeling (as will be discussed). In those cases where a sufficient number of cells could be analyzed, the $V_{\alpha}8$ staining pattern was found to be a stable trait for individual mice, even after boosting (Fig. 4 and data not shown). It is noteworthy that mice displaying a single level of $V_{\alpha}8$ staining were never found to acquire a second subpopulation.

$V_{\alpha}8$ expression in phenotypically distinct subsets of antigen-selected cells found late in the CW3 response

The phenotype of antigen-selected cells from long-term immune mice appears to be more complex than that found early in the CW3 response (8). This is confirmed in the present study in which we still observe a moderate but significant enrichment of $V_{\beta}10^+$ cells among CD62L⁻CD8 cells at day 350 in most mice (mean values of 17.1 and 10.5% for immune and age-matched control mice, respectively; $P < 0.05$, Mann-Whitney test). However, by this stage of the response, there is also a marginal $V_{\beta}10$ enrichment in the CD62L⁺

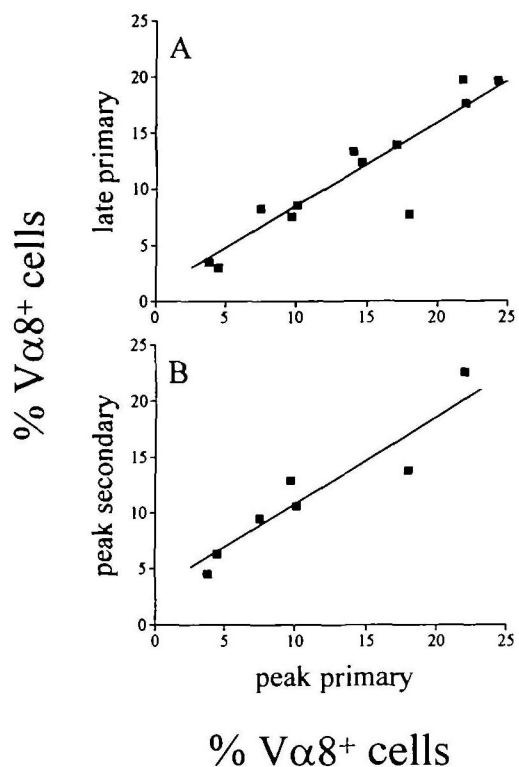


Fig. 3. Stability of individual TCR repertoires selected by antigen during the primary response. The proportion of V β 10⁺CD62L⁻CD8 cells that express a V α 8 TCR was determined by four-color flow cytometry for individual mice at the peak of the primary response, and compared with that found either (A) late in the primary response (day 35) or (B) at the peak of the secondary response (day 11 after a boost given 40 days after the primary immunization). The results were analyzed using linear regression. The line of best fit is indicated for each plot, with correlation coefficients of $r = 0.915$ ($P < 0.00005$) for (A) and $r = 0.941$ ($P < 0.002$) for (B).

subset (mean values of 16.4% for immune mice and 7.4% for control mice; $P < 0.01$, Mann-Whitney test). Even with these modest enrichments for V β 10⁺ cells, we observed that a number of mice (nos 7, 14, 16, 18, and 22 in Table 1) clearly show elevated levels of V α 8 usage among V β 10⁺ cells in both of these subsets. It is noteworthy that these are the same animals which consistently displayed enhanced proportions of V α 8 usage among antigen-selected cells earlier in the response. Furthermore, even after a subsequent boost, the V α 8 repertoire among V β 10⁺ cells remained relatively stable in both subsets defined by the CD62L marker. However, as we had found earlier (8), a further expansion of V β 10⁺ cells was observed only in the CD62L⁻ subset after the boost, to $69.6 \pm 14.2\%$ in this experiment, with almost no change ($17.1 \pm 5.5\%$) among CD62L⁺ cells.

Discrete patterns of high and low level V α 8 expression that were found early in the CW3 response in the CD62L⁻ subset were also evident in long-term immune animals in both the CD62L⁻ and CD62L⁺ subsets (data not shown). Where evident, this pattern of V α 8 expression remained characteristic of an individual animal throughout this long period of analysis, and the same pattern was found in both the CD62L⁻ and

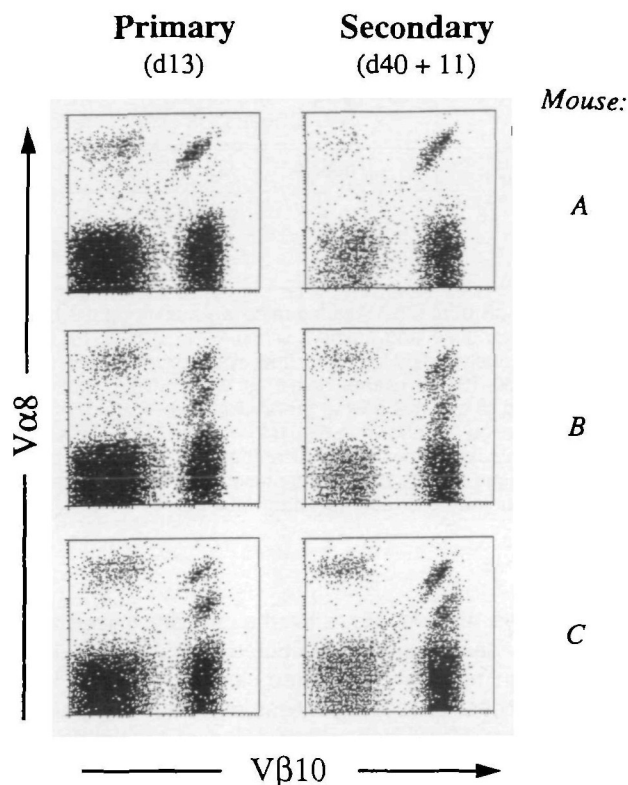


Fig. 4. Antigen-selected cells from individual mice display distinctive V α 8 staining patterns that are conserved at different stages of the CW3 response. PBL are from mice injected with P815-CW3 and bled at the peak of the primary response (day 13), then boosted at day 40 and bled at the peak of the secondary response 11 days later. After staining with mAb to CD8, CD62L, V β 10 and V α 8, PBL were analyzed by four-color flow cytometry. The dot plots show cells gated as CD62L⁻ and CD8⁺, with fluorescence plotted on a log₁₀ scale. The results from individual mice A, B and C are represented to illustrate typical patterns of V α 8 staining.

the CD62L⁺ subsets where V α 8 expansion occurred. These findings add further weight to our interpretation that the CW3-specific cells present in the CD62L⁺ subset in long-term immune mice are derived from the same pool of precursors as those in the reciprocal (CD62L⁻) subset and indeed as those found early in the response.

Calculation of repertoire size for individual mice during the CW3 response

The stability of the V α 8 TCR repertoire observed over time argues against technical error as an explanation for the variability of V α 8 usage among individuals during the CW3 response. If the CW3 repertoire consisted of relatively few clones, such variability would be expected, and the observed mean V α 8 usage and SD for each group of animals could be used to estimate the average repertoire size, as detailed in Methods. By this method, the repertoire for the primary responses shown in Fig. 1 would contain ~15–30 anti-CW3 clones (Table 2). Little change is evident upon boosting. Estimations of repertoire size were also made for a group of mice (nos 7–16 of experiment 3, shown in Table 1) that received booster immunizations of P815-CW3 transfectant

Table 2. Estimates of the size of the CW3-specific V β 10 TCR repertoire

Experiment	No of mice	% V α 8 ⁺ cells among V β 10 ⁺ CD62L ⁻ CD8 cells		Estimated repertoire size
		mean	SD	
1	16	13.3	6.6	26
2	14	11.7	7.8	17
3	16	8.6	7.6	14

PBL from individual CW3-immune mice were taken at the peak of the primary response and stained with mAb to V α 8, V β 10, CD62L and CD8 and analyzed by four-color flow cytometry. For each animal, a total of 5000–10,000 events gated as V β 10⁺CD62L⁻CD8⁺ cells were analyzed to determine what proportion were V α 8⁺. The results for individual mice are shown in Fig 1(A). For each experiment (1, 2 and 3), the calculated mean and SD of the V α 8⁺ cells were used to estimate the number of V β 10⁺ clones present in the gated population of antigen-selected cells (as described in Methods)

cells on days 45 and 357 after the primary immunization. Calculations based on data collected on days 56 and 367 from these animals gave estimated clone numbers of 12 and 13 V β 10⁺ clones respectively. These estimates compare well to a repertoire of 16 clones calculated at the peak of the response (days 14) for the same group of mice. However, one must keep in mind that our calculations are based on the assumption that all clones are of equal size, which may be a simplification of the real repertoire. In fact, our data would also be compatible with a slightly smaller number of highly abundant clones and additional minor clones.

Discussion

Our results demonstrate that V α 8 usage in the CW3 response is highly variable among individual mice, yet relatively stable over time. In contrast, we found little variation in the overall V α 8 usage among V β 10⁺CD8 cells in non-immune controls. Each individual responder animal displayed a characteristic repertoire that was established by an early time point in the primary response and persisted at a relatively stable equilibrium for a significant proportion of the murine lifespan (at least 1 year), even throughout a secondary and tertiary response. While others have documented the long-term persistence of CD8 clones (2–5), ours is the first study to demonstrate that the overall TCR repertoire selected by antigen at an early stage can remain stable for a long period of time. It will now be important to extend this approach to study responses to other antigens. In a recent analysis of the I-E^k-restricted response to pigeon cytochrome *c* (14), it appeared that some selection for particular amino acids occurred between the primary and secondary responses. However, in that study separate pools of animals were used for the two different time points since the animals had to be killed to obtain the lymph nodes. In the CW3 response antigen-selected cells are readily detected among PBL allowing longitudinal analysis of individual mice. An important consideration for vaccine development concerns the possible recruitment of previously activated T cells upon challenge

with heterologous or cross-reactive antigens (15). The availability of simple methods for direct *ex vivo* longitudinal TCR repertoire analysis should open new approaches to investigate these issues.

The stability of the CW3-selected repertoire was further substantiated by our observation that the characteristic staining patterns with mAb against V α 8 found among the mice were conserved throughout the response and after repeat immunizations. It is possible that the V α 8^{dim} population expresses TCR with a lower binding affinity with the mAb, perhaps a sub-family of V α 8, in which case this result can be considered as an independent confirmation of repertoire stability using a different TCR structural marker. The bimodal staining was not the result of insufficient antibody, since increased antibody concentration had no effect on the staining pattern. We consider this phenotype to be compatible with the expression of a second V α chain. Indeed, similar staining patterns were reported in cells from normal human PBL that simultaneously expressed V α 2 and V α 24 (16), and recently the presence of dual α chain-expressing T cells was also demonstrated in the normal murine repertoire (17). However, from these studies it was not known whether T cells expressing two TCR heterodimers at the cell surface could participate in normal *in vivo* antigen-specific responses. In fact, it was proposed that dual α chain T cells may be at a selective disadvantage from being unable to generate as high an avidity as single α chain T cells, due to their lower density of antigen-specific TCR molecules (17). In contrast, our experiments with normal mice suggest that cells expressing two V α chains are indeed selected by antigen during an *in vivo* response.

We suggest that the variability in V α 8 usage found in this study among individual responder mice is most likely due to the oligoclonality of the CW3 response. The stability of individual repertoires with time argues that the level of V α 8 expression is a bona fide characteristic of each responder mouse and that the variations among individuals are not due to technical error or to the inclusion of too few events during the flow cytometric analysis. Importantly, the V α 8 repertoire appears to be skewed only among the expanded CW3-selected T cells, since the proportion of V α 8 cells among CD62L⁺ cells of the same immune animals early in the response, or among V β 10⁺CD8 cells in unprimed, normal mice showed little variability. If only a limited number of V β 10 clones were expanded during the CW3 response, each mouse would not be expected to express exactly the same number of V α 8-expressing clones. Indeed, one can then use the mean and SD observed for V α 8 expression to estimate the average number of V β 10 clones expanded per responder mouse, with the caveats mentioned in the Results section. By this method, we estimate that the CW3-specific repertoire size for individual mice varies from ~15 to 30 clones. This corresponds well to other estimates of repertoire size that we have made sequencing the V β 10–J β 1.2 TCR of single V β 10⁺CD62L⁻CD8 T cells sorted by FACS from CW3-immune mice. In that study we assumed that ~60% of the CW3-specific TCR use the J β 1.2 gene segment, as had been found for CTL clones (10). We then estimated that the size of the CW3-specific repertoire ranged from eight to 35 V β 10⁺ clones per individual. Thus, using two completely independent approaches to assess

TCR expression on antigen-selected cells directly *ex vivo*, one based on PCR amplification and sequencing of the β -chain (9) and the other (this present study) based on flow cytometric analysis of $V_{\alpha}8$ expression, we arrive at very similar estimates for the size of the $V_{\beta}10$ TCR repertoire in the CW3 response. As these are the first studies, to our knowledge, that attempt to calculate repertoire size in individual animals, it will now be interesting to determine repertoire size in other responses.

Compared with the large number of potential $\alpha\beta$ TCR available to the immune system (up to 10^{15} , discussed in 18), the potential size of the CW3-specific repertoire (considering all possible responder mice) appears to be relatively limited, possibly comprising no more than 10^4 different TCR (7) and the size for an individual may be even smaller. An interesting question that arises from our findings is whether the oligoclonality observed reflects the size of the pre-immune repertoire or only a proportion that might be selected in the response. If the initial exposure to antigen activates only a proportion of all possible CW3-specific precursors, those few clones that are stimulated early in the response may subsequently come to dominate the repertoire due to their rapid cycling time (19,20). Such a 'rare-encounter' model has been proposed in explaining the individual clonal dominance to a B cell determinant (21) and in CD8 responses to tumor cells expressing multiple antigens (22). In monitoring CD62L expression on CW3-selected cells, we have made the intriguing observation that phenotypically distinct subpopulations of expanded $V_{\beta}10^{+}CD8$ cells found late in the response apparently express similar TCR repertoires. Previous *in vitro* studies of murine concanavalin A blasts (23) and more recently of *in vivo* activated murine CD8 cells (24,25) have suggested that CD62L may be re-expressed on primed cells late in a response, but direct *in vivo* evidence for CD62L re-acquisition was not demonstrated. Alternatively, it has also been suggested that some virus-specific CTLp may fail to lose expression of CD62L upon initial stimulation by antigen (25). While indirect, the data described herein provide strong *in vivo* evidence that CD62L⁺ antigen-selected cells from long-term immune mice are actually derived from precursors expressing the same TCR repertoire as the CD62L⁻ cells, making CD62L re-expression *in vivo* the most probable explanation. It should now be possible to test this more directly by sequencing sorted cells. It has recently been proposed that events leading to the possible re-expression of CD62L on activated cells may be greatly influenced by differences in affinity of individual TCR (25). Our results suggest that, at least in the CW3 response, TCR affinity would not play a major role at this level of selection, since similar TCR repertoires appear to be expressed in both CD62L⁻ and CD62L⁺ subsets.

Our results suggest that, at least in the CW3 response, little further evolution of the TCR repertoire occurs after the peak of the primary response, even in mice boosted up to 1 year later. Taking advantage of the CW3 system where antigen-selected cells can be analyzed *ex vivo* in normal mice, we recently demonstrated that substantial changes occur in the level of expression of the TCR and co-receptor molecules at different stages of the response (8). It is possible that such changes modify the overall avidity of T cell-target cell inter-

actions and could thus play an important role in the functional evolution of T cell responses once the initial selection of the TCR repertoire has occurred early in the primary response.

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Abbreviations

CTL	cytotoxic T lymphocyte(s)
PBL	peripheral blood lymphocyte(s)

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