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Individual Variations in the Murine T Cell Response to a Specific Peptide Reflect Variability in Naive Repertoires

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Summary

Previous studies have analyzed the diversity of T cell responses upon immunization. Little is known, however, about the individual variability of naive repertoires and its influence on immune responses. In the present study, T cells specific for a K^d-restricted epitope derived from HLA-A2 were purified from individual immunized mice using tetramers of MHC–peptide. Their TCRβ chains were sequenced revealing strong biases but large variations in BJ usage and clonal composition. Most importantly, sequence analysis from nonimmunized mice demonstrated the preexistence of a small set of splenic precursors, distinct in each mouse and comprising less than 200 cells. Therefore, differences in precursor pools appear to be the major source of individual variability in antigen-selected repertoires.

Introduction

CD8 αβ T lymphocytes recognize short peptidic fragments presented at the surface of antigen-presenting cells by major histocompatibility complex (MHC) class I molecules. Recognition occurs via the T cell receptor (TCR) that confers the antigenic specificity upon individual T cells (Dembic et al., 1986). TCRs are heterodimers made of α and β chains that arise from the imprecise juxtaposition of V, (D), and J gene segments and the addition of template-independent N nucleotides during this process (Alt et al., 1992). Assuming this recombination process occurs randomly, the theoretical diversity of the murine αβ TCR has been calculated to be of the order of 10^{15} (Davis and Bjorkman, 1988). This number is much higher than the actual size of the peripheral T cell compartment, estimated to be 10^8 cells. However, the theoretical diversity has been overestimated since (1) biases in the recombination machinery have been documented (Candeias et al., 1991), (2) β chain is first selected independent of the α chain (Mallick et al., 1993; Dudley et al., 1994), (3) structural constraints operate on the pairing of the α and β chains (Malissen et al., 1988; Saito et al., 1989; Kuida et al., 1991), and (4) the T cell repertoire bears the imprint of positively selecting self-peptides (Sant'Angelo et al., 1997) and is narrowed by negative selection in the thymus (van Meerwijk et al., 1997). Thus, the actual complexity of the mature repertoire is not known, and the extent to which T cell repertoires of distinct individuals overlap has not yet been elucidated.

The size and the diversity of the set of naive T cell precursors may be critical in shaping the immune response against a given antigen. The question of the extent to which individual variability in the naive repertoires affects the magnitude and complexity of antigen-specific responses has been difficult to address. On the one hand, T cell precursors in nonimmunized animals are difficult to detect and characterize because of their low frequencies. On the other hand, little is known about the global composition of antigen-selected repertoires in immune animals. Most studies have relied on the analysis of CTL clones or T cell hybridomas (Casanova and Maryanski, 1993), which provides only partial information on the complexity of immune repertoires in individual responders. In the case of T cell responses displaying a limited set of TCR chains, antigen-specific cells have been isolated using a combination of anti-TCR V region antibodies together with cell surface activation markers (McHeyzer-Williams and Davis, 1995; Maryanski et al., 1996), but this approach focused on a fraction of the specific response only.

In this study, we analyzed the TCRβ repertoires specific for the well-characterized antigenic peptide derived from HLA-A2 and presented by K^d (Casanova et al., 1993). Using two original approaches, we studied the relationship between the antigen-specific T cell repertoire in immunized and naive mice. We took advantage of a recent technique that uses tetramers of MHC–peptide complexes to label and isolate specific T cells (Altman et al., 1996). We then exhaustively sequenced TCRβ chains of antigen-specific T cells in three immune animals. Small and distinct sets of TCR sequences were identified in each animal. To understand the origin of this variability, we sequenced the same BV-BJ joints in naive animals. This enabled us to estimate the upper limit of the number of specific T cell precursors at about 200 cells per spleen in naive animals. We found that more than 75% of the TCRβ chains were unique to each nonimmunized mouse. We conclude from these results that the diversity of the immune repertoire in an individual mouse is strongly limited by the size of the naive precursor pool before immunization. Therefore, the individual variability observed in the clonal composition of the immune response reflects large variations in the naive repertoires of distinct animals.

Results

K^d-Peptide Tetramers Identify All Antigen-Specific T Cells

It has been previously shown that the immune response against the K^d-restricted epitope 170–179 derived from

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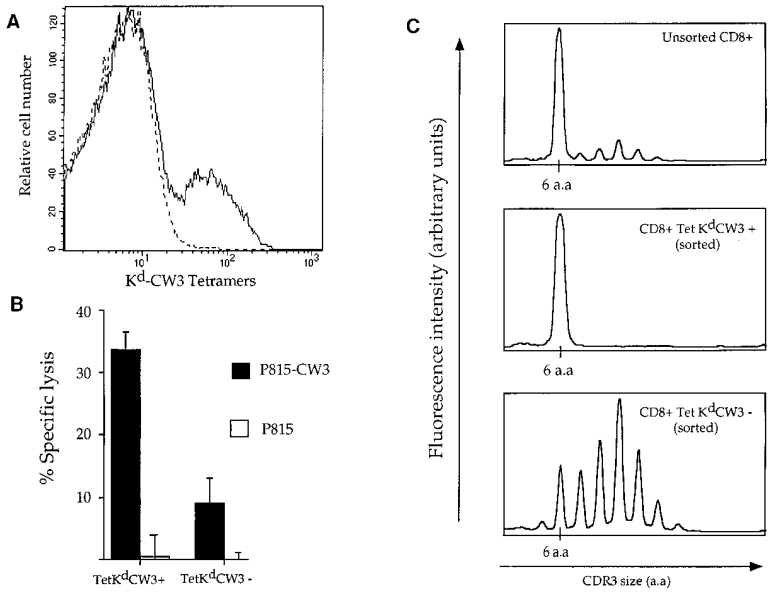

Figure 1. K^d-CW3 Tetramers Bind CW3-Specific T Cells

(A) Specific binding of K^d-CW3 tetramers. DBA/2 mice were injected i.p. with 10^7 P815 (dotted lines) or 10^7 P815-CW3 (solid lines) tumor cells. Ten days postinjection, splenocytes were double stained with an anti-CD8 antibody and K^d-CW3 tetramers (histograms are gated on $CD8^+$ cells). In the immunized mouse, 20% of the $CD8^+$ T cells were stained with the tetramers.

(B) Ex vivo cytolytic activity of tetramers bound $CD8^+$ T cells. Sorted $CD8^+TetK^dCW3^+$ and $CD8^+TetK^dCW3^-$ splenocytes were assayed for cytotoxicity on ^{51}Cr-labeled P815-CW3 or P815 target cells in 4 hr ^{51}Cr-release assay. Effector:target ratio was 7 to 1. Results are means of duplicates.

(C) CDR3β size distribution among $BV10^+$ splenocytes from total $CD8^+$ and sorted $CD8^+TetK^dCW3^+$ and $CD8^+TetK^dCW3^-$ populations. cDNA from the indicated cell populations was subjected to PCR using BV10- and BC-specific primers followed by a runoff with a nested fluorescent BC-specific primer. The size distribution was analyzed with the Immunoscope software. Comparison of the surface areas of the different peaks indicated that the contributions of the T cells displaying a 6 aa long CDR3β were 75%, 100%, and 12% in the top, middle, and bottom panels, respectively.

HLA-CW3 (CW3) is very limited in terms of TCR usage and that CW3-specific T cells use exclusively the *TCRBV10* gene segment with a 6 amino acid (aa) long CDR3β (Casanova et al., 1992). DBA/2 mice were immunized intraperitoneally with either P815 or P815-CW3 tumor cells. After 10 days, splenocytes were stained using K^d-CW3 tetramers and an anti-CD8 antibody. As shown in Figure 1A, mice immunized with P815-CW3 displayed a T cell population labeled with tetramers, whereas no staining was observed in mice immunized with P815.

In order to determine whether all CW3-specific T cells were stained with K^d-CW3 tetramers ($TetK^dCW3$), we used two approaches. We sorted the $CD8^+TetK^dCW3^+$ and the $CD8^+TetK^dCW3^-$ splenocytes 10 days after intraperitoneal injection of 10^7 P815-CW3 tumor cells. These populations were first assayed for cytolytic activity on P815-CW3 cells. As shown in Figure 1B, sorted $CD8^+TetK^dCW3^+$ cells specifically killed P815-CW3 tumor cells even at a low effector:target ratio, whereas no significant killing was observed in the $CD8^+TetK^dCW3^-$ population. We then compared the CDR3β size pattern distribution by the Immunoscope technique in the two sorted populations (Pannetier et al., 1995). As shown in Figure 1C, $CD8^+$ splenocytes from P815-CW3 injected mice displayed an expansion corresponding to the CW3-specific response (TCRBV10, 6 aa long CDR3β), which could already be detected before sorting with the tetramers. This expansion was associated only with the $CD8^+TetK^dCW3^+$ sorted population, whereas it was almost absent from the $CD8^+TetK^dCW3^-$ cells. By comparing the surface area of the different CDR3β size peaks (Figure 1B), we calculated that about 97% of the specific T cells were stained with the tetramers.

Before sorting, 30% of all $CD8^+$ T cells were $BV10^+$. From the Immunoscope profile (Figure 1B, top panel), we calculated that about 75% of the $CD8^+BV10^+$ cells had a CDR3β of 6 amino acids. Therefore, the expected frequency of the CW3-specific cells among the $CD8^+$ should be 30% $\times$ 75% = 22.5%, in good agreement with the tetramer staining (20%).

Magnitude and Fine Specificity of the A2-Specific T Cell Response

The A2-derived epitope differs from CW3 by a single amino acid substitution at position 173 (Martinon et al., 1989). It has been shown previously that among the A2-specific T cell clones, some recognize CW3, while others do not, indicating that there are at least two patterns of peptide recognition (Casanova et al., 1993). To investigate the contribution of these two components in individual A2-specific responses, we prepared tetramers of biotinylated K^d-CW3 and K^d-A2 complexes. Five DBA/2 mice were injected intraperitoneally with 10^7 P815-HLA-A2 transfected cells. After 2–3 weeks, splenocytes were triple stained with an anti-CD8 antibody, an anti-CD62L (Mel14) antibody, and K^d-peptide tetramers. $CD8^+TetK^dA2^+$ as well as $CD8^+TetK^dCW3^+$ cells expressed low levels of CD62L, indicating that they had been activated (Figure 2). The ratio of the $CD8^+TetK^dCW3^+$ subset to the $CD8^+TetK^dA2^+$ subset was relatively constant in the five mice studied (50%, 45%, 75%, 60%, and 55%; mean value of 57%) despite some variations in the percentage of the A2-specific cells among the $CD8^+$ (0.8%, 9.4%, 8.4%, 5.0%, and 11.2%). This variation, as we and others have observed, was in fact mostly related to differences in kinetics. Thus, it is likely that mouse 1 was sacrificed after the peak of the immune response had been reached. The four other mice responded with close magnitudes (8.5% $\pm$ 2.3%). Taken together, these results demonstrate a recurrent and quite similar contribution of both patterns of recognition in the A2-specific response.

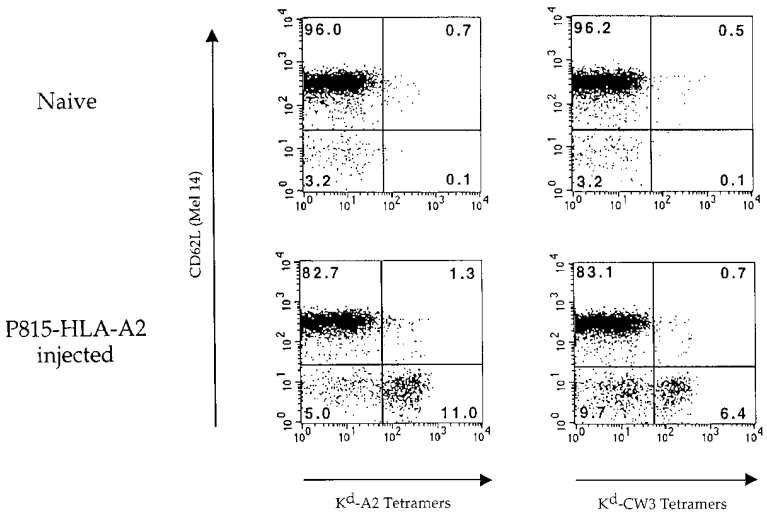

Figure 2. Phenotypic Analysis of A2-Specific T Cells and Cross-Reaction with CW3

Left panels: most of the A2-specific T cells express low levels of CD62L. DBA/2 mice were injected i.p. with 10^7 P815-HLA-A2 tumor cells. After 3 weeks, splenocytes were triple stained with anti-CD8, anti-CD62L (Mel14), and K^d-A2 tetramers (dot plots are gated on $CD8^+$ cells). Numbers shown are the percentage of cells that fall into indicated quadrant.

Right panels: a large fraction of the A2-specific T cells cross-reacts with CW3. The same cells as in the left panels were triple stained with anti-CD8, anti-CD62L(Mel14), and K^d-CW3 tetramers.

TCRV Usage in the Antigen-Specific Repertoire of Individual Mice

In order to characterize TCRβ rearrangements used in these two T cell subsets, we purified the $CD8^+TetK^dA2^+$ cells from three different P815-HLA-A2 immunized mice and the $CD8^+TetK^dCW3^+$ cells from two of these mice (mouse 1 did not yield a sufficient number of $CD8^+K^dCW3^+$ cells). Semiquantitative analysis of TCRBV transcripts was performed on these different samples. In order to identify BV segments that were significantly used, we compared for each BV segment the relative abundance of TCRV transcripts in the sorted cells and in a population of $CD8^+$ splenocytes purified from an age-matched naive DBA/2 mouse. As shown in Figure 3, BV10 segment usage was drastically increased (12- to 16-fold) in all sorted samples compared to the naive population. In contrast, other TCRBV transcripts were significantly below background. We conclude from these results that both $CD8^+TetK^dA2^+$ and $CD8^+TetK^dCW3^+$ populations expressed mainly, if not exclusively, the *BV10* gene segment.

Using the same approach, we measured the relative abundance of TCRAV8 segment in A2- and CW3-specific T cells. This segment was expressed in all sorted samples (Figure 3). However, in the $CD8^+TetK^dA2^+$ and $CD8^+TetK^dCW3^+$ subsets isolated from mouse 2, TCRAV8 was more frequent than in mouse 3, indicating individual variability in the AV8 segment usage among the specific populations.

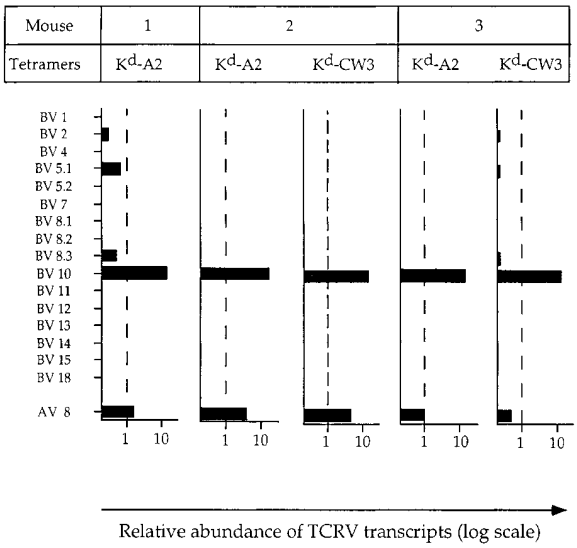

Figure 3. Relative Abundance of TCRV Transcripts in A2-Specific T Cells

A2-specific lymphocytes mainly use the BV10 segment. Mice 1–3 were injected i.p. with 10^7 P815-HLA-A2 tumor cells. After 3 weeks, splenocytes were stained with an anti-CD8 antibody and either K^d-A2 or K^d-CW3 tetramers. $CD8^+TetK^dA2^+$ and $CD8^+TetK^dCW3^+$ populations were sorted ($CD8^+TetK^dCW3^+$ were not sorted from mouse 1 due to the low number of cells). The relative abundance of the different TCRBV and of TCRAV8 transcripts was determined by semiquantitative PCR as described in Experimental Procedures. The values obtained for $CD8^+$ splenocytes of a naive mouse were set to 1 and used to normalize those obtained for the sorted splenocytes of immunized mice 1–3.

CDR3β Length Distribution in Antigen-Specific Repertoires of Individual Mice

We further characterized the TCR of the sorted cell populations with respect to their CDR3β length distribution, using the Immunoscope technique. To evaluate the sensitivity of our approach, we took advantage of the aforementioned low-responder mouse 1, in which 0.8% of $CD8^+$ T cells (less than 0.3% of the T cells) were stained with K^d-A2 tetramers. As expected, the TCRBV10-CDR3β profile of the unsorted $CD8^+$ splenocytes did not show any major deviation from the Gaussian-like profile found in naive animals (Figure 4A). However, minor expanded populations, which slightly disturb this profile (indicated by arrows in Figure 4A), were purified in the $CD8^+TetK^dA2^+$ sorted cells while they were absent from the $CD8^+TetK^dA2^-$ population. They displayed a 6 or 10 aa long CDR3β, as previously described for a panel of A2-specific CTL clones (Casanova et al., 1993). Therefore, it is possible to purify and characterize small T cell subsets by combining tetramer-based sorting and Immunoscope analysis.

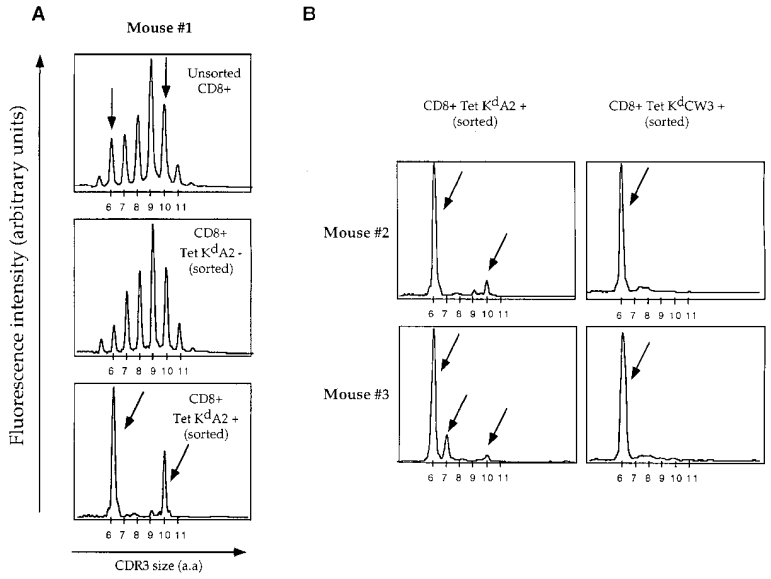

Figure 4. CDR3β Size Distribution among Antigen-Specific T Cells

(A) Tetramer-based sorting allows the purification of small expanded T cell populations. Immunoscope analysis was performed as described in the legend of Figure 1B on unsorted $CD8^+$ (top) and on sorted $CD8^+$TetK^{d}A2$^-$ (middle) and $CD8^+$TetK^{d}A2$^+$ (bottom) populations from mouse 1. The arrows mark the two peaks (6 and 10 amino acid) corresponding to the A2-specific lymphocytes.

(B) Individual analysis of the TCRBV10-CDR3β length usage among antigen-specific populations. Antigen-specific T cells were sorted from mice 2 and 3 after staining with an anti-CD8 antibody and the indicated tetramers. Immunoscope analysis was performed as above. Arrows indicate expansions discussed in the text. While A2-specific cells display CDR3β of several lengths, the subset that cross-reacts with CW3 displays only 6 aa long CDR3β.

The same lengths of CDR3β were also found in the two other mice (Figure 4B). The relative contribution of the two CDR3β sizes varied in different animals. Moreover, some A2-specific T cells derived from mouse 3 displayed a 7 aa long CDR3β. Finally, TetK^{d}CW3$^+$ sorted cells exclusively used 6 aa long CDR3β (Figure 4B). Taken together, these results indicate a variable usage of preferential CDR3β lengths.

Sequences of TCRβ Rearrangements Associated with K^d-A2 Recognition

In order to refine the molecular analysis of A2-specific T cells, we cloned and sequenced the TCRβ chains expressed by specific T cells from the three mice. Since all sorted cells were shown to use exclusively the TCRBV10 segment, we amplified their cDNA by PCR using a BV10- and a BC-specific primer. After cloning into *E. coli*, individual nucleotide sequences were determined. As more and more sequences were obtained, the number of distinct sequences reached a plateau, suggesting that the vast majority of the response had been described.

A total of 182 colonies were sequenced from five sorted T cell populations. The deduced CDR3β amino acid sequences are listed in Table 1. The 182 sequences represented 27 distinct nucleotide sequences, all displaying the SXG motif known to be strongly selected in the A2 response (Table 1). Most sequences were highly homologous or identical to those of previously reported CTL clones (Casanova et al., 1993). Each amino acid sequence corresponded to a single nucleotide sequence, except for one CDR3β (SYGSDY) from mouse 2. The most frequent sequences were STGNTL in mouse 1 (40%), SWGSDY in mouse 2 (31%), and STGQRY in mouse 3 (31%). As expected, almost all sequences (95%) from the $CD8^+$TetK^{d}CW3$^+$ cells were found in $CD8^+$TetK^{d}A2$^+$ cells, except for two that were found at low frequency (2/40 and 1/31) in the $CD8^+$TetK^{d}CW3$^+$ subset. The simplest explanation for this result lies in the smaller number of rearrangements present in the $CD8^+$TetK^{d}CW3$^+$ subset, which allowed the detection of rare CDR3β. The CDR3β size analysis presented in the previous section (Figure 4B) was in total agreement with the sequences obtained.

Sequences from lymphocytes that cross-react with CW3 exclusively displayed a 6 aa long CDR3β predominantly associated with the BJ1.2 segment (Table 1). Non-cross-reacting cells also used predominantly the 6 aa long CDR3β in preferential combination with other BJ segments (2.4 for mouse 2, 1.3 for mouse 3). Longer CDR3β were found as well (7 aa long associated with BJ1.2, 10 aa long associated with BJ1.4). Figure 5 (left panel) summarizes, in a separate matrix for each mouse, the contribution of the different CDR3β size/BJ segment combinations to the A2-specific response. From the staining and the sequence data, we determined the clonal composition of the A2-specific response: absolute numbers of A2-specific T cells bearing the various CDR3β sequences are shown in Figure 5 (right panel). In the three analyzed mice, more than 88% of the response is contributed by less than six CDR3β sequences, some of which are displayed by up to 4×10^5 T cells.

Peripheral β Chain Repertoire of Naive Mice Displays Important Individual Variations

To estimate the size and the individual variability of the A2-specific repertoire in naive mice, we purified splenic $CD8^+$ T cells from two nonimmunized animals raised in SPF conditions and sequenced 6 aa long CDR3β regions from BV10-BJ1.2 rearrangements. As explained in Experimental Procedures, cDNA was amplified using BV10- and BJ1.2-specific primers. PCR products with a 6 aa long CDR3β were separated by electrophoresis before cloning and sequencing. To assess the reproducibility of this technique, the experiment was performed in duplicate for mouse A. Total splenocytes were split into two samples (A and A′). CD4 depletion, PCR, and cloning were done independently. Among 92 nucleotide sequences obtained from sample A′, 82% were identical to some of the 215 sequences from sample A. This

Table 1. CDR3β Amino Acid Sequences from Sorted Samples

	$CD8^+$ $TetK^d$ $A2^+$					$CD8^+$ $TetK^dCW3^+$				
	BV10	CDR3	BJ	BJ Segment	Number[a]	BV10	CDR3	BJ	BJ Segment	Number[a]
Mouse 1	CAS	STGNTL	YFG	1.3	6/15					
	CAS	SYGSDY	TFG	1.2	2/15			not done		
	CAS	SLGSRY	TFG	1.2	1/15					
	CAS	STGTGPNERL	FFG	1.4	6/15					
Mouse 2	CAS	SWGSDY	TFG	1.2	16/51	CAS	SWGSDY	TFG	1.2	26/40
	CAS	SRGNTL	YFG	2.4	14/51					
	CAS	SRGSDY	TFG	1.2	6/51	CAS	SRGSDY	TFG	1.2	5/40
	CAS	SYGSDY	TFG	1.2	6/51	CAS	SYGSDY	TFG	1.2	4/40
	CAS	SFGITQ	YFG	2.5	3/51					
	CAS	SFGQEV	FFG	1.1	2/51					
	CAS	SYGPDY	TFG	1.2	1/51					
	CAS	SYGSDY	TFG	1.2	1/51	CAS	SYGSDY	TFG	1.2	3/40
	CAS	SLGSDY	TFG	1.2	1/51					
	CAS	STGTGGNERL	FFG	1.4	1/51					
						CAS	SRGVEQ	YFG	2.7	2/40
Mouse 3	CAS	STGQRY	TFG	1.2	14/45	CAS	STGQRY	TFG	1.2	19/31
	CAS	SSGNTL	YFG	1.3	8/45					
	CAS	SSGQDY	TFG	1.2	6/45	CAS	SSGQDY	TFG	1.2	10/31
	CAS	SFGQTY	TFG	1.2	3/45					
	CAS	SRGNTL	YFG	2.4	2/45					
	CAS	SLGSDY	TFG	1.2	1/45					
	CAS	SLGQEV	FFG	1.1	1/45	CAS	SLGQEV	FFG	1.1	1/31
	CAS	SLGQGNY	TFG	1.2	3/45					
	CAS	SSGGSPL	YFA	1.6	3/45					
	CAS	SRGASDY	TFG	1.2	3/45					
	CAS	SFGTGLNERL	FFG	1.4	1/45					
						CAS	SLGLKQ	FFG	2.1	1/31

[a] Sequence occurence/total number of sequences. Identical amino acid sequences derived from different nucleotide sequences are compiled separately.

observation demonstrates that the 215 sequences from sample A represent an almost complete description of the naive T cell subset and that the whole procedure is reproducible.

On mouse B, 130 sequences were performed. As more sequences were obtained from mice A and B, the number of distinct CDR3β sequences in each mouse reached a plateau (data not shown), confirming that most of the analyzed T cell subset had been sequenced. Nucleotide sequences encoded 66 distinct amino acid sequences in mouse A and 44 in mouse B. Out of these 110 amino acid sequences, 101 were encoded by a single nucleotide sequence (data not shown). CDR3β sequences from mice A and B are listed in Figure 6. Most sequences

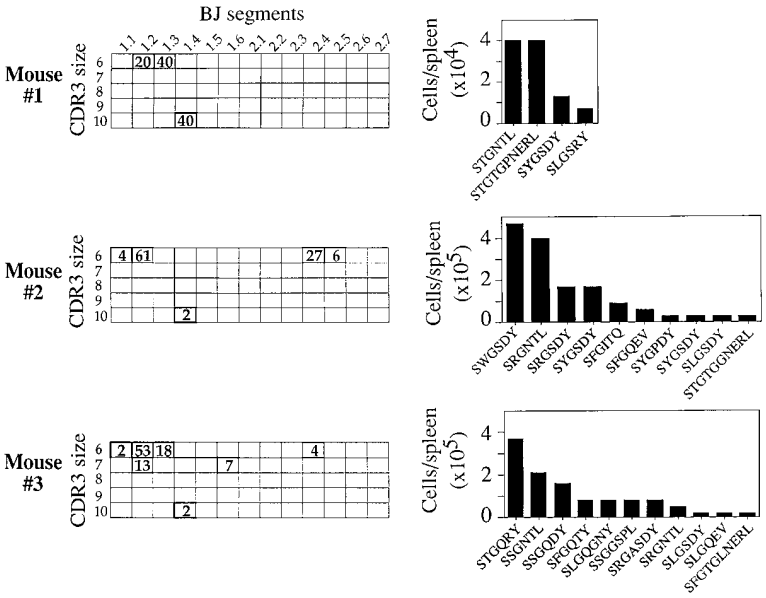

Figure 5. Composition of the A2-Response in the Spleen of Immune Mice

Left panel: individual variability in the composition of the A2-specific T cell response. For each mouse, TCRβ sequences listed in Table 1 are analyzed for their BJ usage and CDR3β length. The numbers indicate the relative contribution of each CDR3β length/BJ segment combination among the A2 response. Empty boxes indicate that the corresponding combination is not observed.

Right panel: clonal composition of the immune response. The absolute number of A2-specific T cells isolated from mice 1–3 and bearing the indicated CDR3β sequence was calculated from the tetramer staining experiment and from the sequence data listed in Table 1.

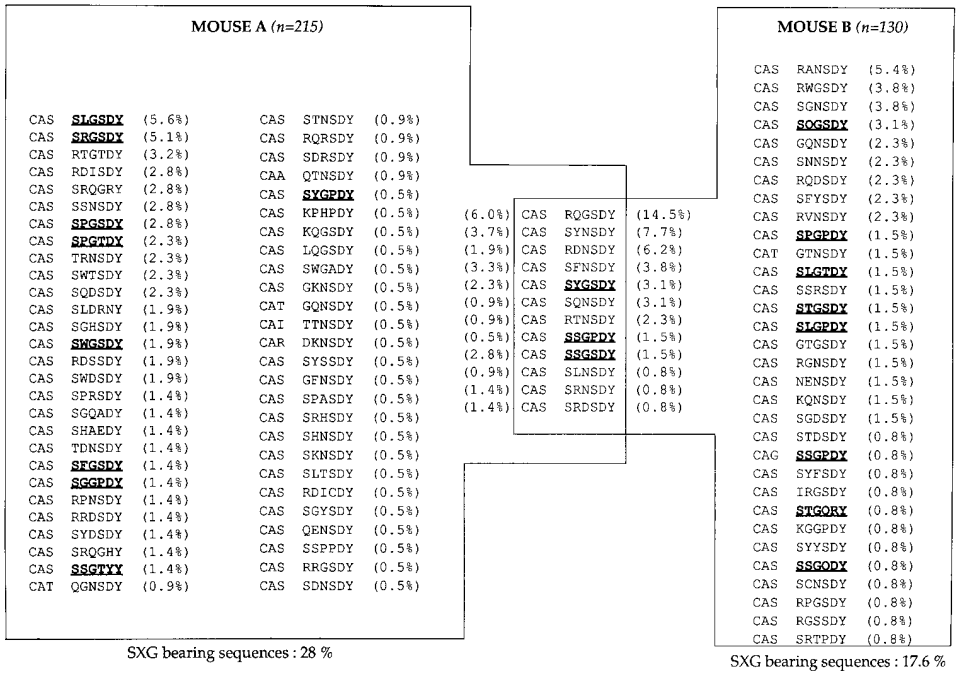

Figure 6. T Cell Repertoires of Distinct Naive Animals Are Mostly Nonoverlapping

Splenocytes from two naive DBA/2 mice (mice A and B), were depleted of $CD4^+$ cells. cDNA was amplified using BV10- and BJ1.2-specific primers. PCR products were run on an 8% polyacrylamide gel. The band corresponding to the 6 aa long CDR3β was cut out from the gel. Further purification, cloning, and sequencing were performed as specified in Experimental Procedures. Figure shows the deduced amino acid CDR3β sequences. The percentage of each sequence is indicated in brackets. Distinct nucleotide sequences encoding the same amino acid sequence are compiled together. CDR3β sequences displaying the SXG motif are underlined.

were private, i.e., unique to a given mouse (44/66 and 32/44 for mice A and B, respectively) and only 12 were shared by both animals. Finally, 28% and 18% of the sequences derived from mice A and B, respectively, bore the SXG motif within the CDR3β. Here again, most of these SXG-bearing sequences were private.

In summary, as found in the immune repertoire, the naive repertoire was mostly nonoverlapping in distinct animals.

Discussion

In this report we thoroughly analyzed the size, the diversity, and the individual variability of the A2-specific T cell repertoire in naive and immune animals. Our observations show that each immune mouse displays a unique and small repertoire specific for A2 and that this variability can be accounted for by a small and nonoverlapping set of specific precursors in each animal.

Analysis of Antigen-Specific Repertoire Using Tetramers of MHC–Peptide

The approach used in the present study combined several previously described techniques: (1) staining and purification of specific T cells with tetramers of MHC–peptide complexes, (2) PCR-based quantitation of BV-BC rearrangements (in particular by the Immunoscope method), and (3) sequencing of TCRβ chains. To validate our approach, we ensured that tetramers stained all antigen-specific T cells in the related and very restricted CW3 response. The sensitivity of the method was determined in a weak-responder animal immunized with P815-HLA-A2; the combination of tetramers and Immunoscope enables the purification and the characterization of small T cell populations representing less than 0.3% of the total T cells. Previous studies using Immunoscope or other PCR-based techniques assessed the specificity of T cells by comparing TCR sequences to those of T cell clones of known specificity (Cibotti et al., 1994; Levraud et al., 1995; Maryanski et al., 1996). In the present strategy, the specificity of the analyzed T cells was detected directly by interaction of the TCR with its ligand. Our approach enables a complete and quantitative clonal description of the overall antigen-specific response (Figure 5). Therefore, this approach offers a unique opportunity to characterize the entire response against a given peptide and even to analyze the cross-reactions with related peptides.

The experimental strategy was designed to minimize any distortion in the representation of individual sequences. All PCR amplifications were performed using the same couple of primers. Since T cell activation does not substantially modify the amount of TCR chain transcripts (Regnault et al., 1996), we performed PCR reactions on cDNA rather than on genomic DNA in order to facilitate the detection of all specific TCR rearrangements. All PCR products displayed the same or similar length (373 ± 6 nucleotides). Previous studies have shown that under such conditions PCR biases are minimal (Pannetier et al., 1993b). Similarly, cloning of the

PCR products does not introduce major biases, as the length and the extremities of the PCR products were the same. Comparison of the sequence data (obtained after cloning) and the Immunoscope profiles (obtained before cloning) substantiates this assertion. Moreover, the percentage of A2-specific T cells that cross-recognize CW3 as determined by tetramer staining (i.e, before PCR and cloning) is remarkably close to the percentage of A2-specific sequences found to cross-react with CW3. The reproducibility of the approach was directly demonstrated by analyzing the repertoire of the nonimmunized mouse A in duplicate. For these reasons, we consider that the sequence data presented in Table 1 and Figure 6 represent a faithful picture of the immune and naive repertoire, respectively.

Conserved Magnitude, Complexity, and Fine Specificity of the Immune Response against the A2 Epitope

Analysis of the sequence data shows that in most cases (32/34) within one immune mouse, identical CDR3β amino acid sequences correspond to identical nucleotide sequences, probably reflecting clonal expansions (Table 1). The occurrence of different clones expressing identical TCRβ sequences but different α chains cannot be ruled out; previous analyses of CTL clones have, however, shown this to be infrequent (Casanova et al., 1993). Thus, assuming that identical sequences derived mostly from single clones, more than 88% of the response is contributed by 3, 5, and 6 clones in the different mice.

Maryanski et al. described similar results for the response against P815-CW3 (Maryanski et al., 1996). Interestingly, although TCRβ usage in the A2 response is more diverse in terms of BJ usage and CDR3β length than in the CW3 response (Casanova et al., 1993), this is not reflected by the recruitment of a larger number of clones. In addition, we calculated that the largest clone in mice 1, 2, and 3 contained 4×10^4, 5×10^5, and 4×10^5 cells, respectively. As found recently in a virus-specific response (Muralikrishna et al., 1998), the majority of activated T cells was stained with tetramers (Figure 2), indicating that bystander activation was minimal. Therefore, in this system most antigen-selected lymphocytes derive from a small number of clones that are so efficiently amplified that they represent up to several percent of the peripheral T cell population. Interestingly, the ratio between the frequencies of A2-specific T cells that recognize CW3 and those that do not is well conserved in five analyzed mice. Taken together, these results indicate that various individuals mount a similar response in terms of magnitude, complexity, and cross-reactivity.

Large Individual Variability in the Composition of Immune Repertoires

When individual repertoires are analyzed, conserved features are clearly identified. All A2-specific T cells express the *BV10* gene segment and display the SXG motif in their CDR3β. Individual variability was nevertheless apparent at four levels of resolution. First, the AV8 usage was different in the three mice (Figure 3). Second, mice 1 and 2 used CDR3β of six and ten residues exclusively, but with different ratios, while mouse 3 used CDR3β of six, seven, and ten residues (see Immunoscope profiles in Figure 4). Third, the BJ × CDR3β matrices (Figure 5, left panel) were unique to each mouse, with only two combinations (6×1.2 and 10×1.4) common to all three mice. Fourth, at the level of nucleotide sequences, not one CDR3β sequence was shared by all three mice, and only three sequences were shared by two distinct animals. Moreover, the contribution of these few recurrent sequences to the response was highly variable in the different mice.

Thus, comparison of the immune repertoire in three mice indicates that despite the constraints imposed by antigenic recognition, extensive stochastic variability takes place in the fine composition of the T cell response. Several explanations could account for the observed variability. If the same set of T cell precursors preexists in the different animals, random encounter with the antigen could result in differential expansion of individual antigen-specific clones. Alternatively, individual repertoires could be substantially different before immunization, and the response could be strongly constrained by the distinct set of available naive precursors in each animal.

The A2-Specific Precursor Pool in Naive Animals Is of Small Size

To explore the latter hypothesis, we analyzed the T cell repertoire of two nonimmunized mice. An initial attempt to purify naive T cells using K^d-A2 tetramers failed to detect significant enrichment in A2-specific T cells, suggesting that the frequency of specific precursors was below the background. We circumvent this difficulty by analyzing a splenic subset expected to contain the A2 precursors. Because the most conserved features between the A2- and CW3-specific T cells in the immune mice are BV10, BJ1.2, and CDR3β of six amino acids, we focused our analysis on this particular subset of cells.

About 18% of the CDR3β sequences analyzed from mouse B displayed the SXG motif (28% for mouse A). We used this percentage to evaluate the number of A2-specific precursor T cells in naive animals. Since 0.4% of the splenocytes are $CD8^+BV10^+$ and 5% use the BJ1.2 segment, and since the 6 aa long CDR3β contribution among BV10-BJ1.2 combination is 3.7% (as determined from the surface area of the profile obtained by Immunoscope), we calculated that about 100 cells per spleen express the *BV10* and *BJ1.2* gene segments together with a 6 aa long CDR3β bearing the SXG motif. Thus, 100 cells are possibly specific. This calculation holds irrespective of the TCRα chain sequences and is likely to be an overestimation. Although not all A2-specific T cells express BV10BJ1.2 rearrangements, these cells contributed to more than 50% of the specific immune response in mice 2 and 3. Therefore, no more than 200 A2-specific T cell precursors are present in each spleen, corresponding possibly to 600 cells in the entire mouse. This estimate is significantly lower than that given recently (3000 cells) for an epitope derived from the lymphocytic choriomeningitis virus (Butz and Bevan,

1998). Finally, our calculation demonstrates that the large magnitude of the A2-response is not due to an exceptionally high precursor frequency.

Distinct Syngeneic Naive Animals Do Not Share the Same Precursor Pool

The most remarkable observation is that at least 75% of the naive T cells were unique to each mouse (Figure 6). The same percentage was found by analyzing 117 and 283 sequences in two other DBA/2 mice (data not shown). This finding contrasts with previous studies on naive repertoires that showed a remarkable conservation of several parameters including BV, BJ usage, and CDR3 size distributions among distinct syngeneic animals (Pannetier et al., 1993a; Kato et al., 1994). In the present study, we increased the degree of resolution of repertoire analysis and compared TCRβ rearrangements at the level of amino acid sequences. This enabled us to reveal extensive individual variations. Although this study focuses on a small subset of the repertoire, preliminary data on other BV-BJ rearrangements (A. C., J. K., and P.K., unpublished data) confirm that, at a given time, naive repertoires are significantly distinct in animals that are otherwise genetically identical and raised in similar environments.

The extent to which differences between T cell repertoires may lead to differences in the overall efficiency of immune responses is unknown. On the one hand, individual variations in the naive repertoire may result in the lack of CTL precursor for a given specificity in some animals. Previous studies showed that holes in naive T cell repertoires can dramatically compromise T cell responses (Nanda et al., 1991; Kumar and Sercarz, 1994; Das et al., 1996). On the other hand, the degeneracy of TCR specificities may allow similar functional responses to be mounted from distinct TCR repertoires. Therefore, new tools allowing the detection of naive T cell precursors should help to unravel this question.

General Conclusion

The present study shows that extensive variability is detected in individual immune responses. While the experimental system analyzed here had been previously described as poorly diverse, we found that the vast majority of the TCR sequences is unique to each mouse. We demonstrate that the naive set of precursors is distinct in each mouse and therefore limits the complexity of individual response. Remarkably, the T cell repertoire appears flexible enough to allow similar T cell responses to be contributed by distinct sets of precursors. Whether this capability originates from its large diversity or bears the imprint of the selective processes that shape it remains to be elucidated.

Experimental Procedures

Mice, Cell Lines, and Chemicals

Tumor cell lines P815, P815-HLA-A2, and P815-CW3 transfectants have been described previously (van Pel et al., 1985; Maryanski et al., 1986; Martinon et al., 1989). Peptides corresponding in sequence to region 170–179 of HLA molecules A2 (RYLENGKETL) and CW3 (RYLKNGKETL) were purchased from Neosystem (Strasbourg, France). All mice used in this study were 8-week-old DBA/2 males raised in SPF conditions and obtained from IFFA-Credo (l'Arbresle, France). Immunization were performed by i.p. injection of 10^7 tumor cells.

Preparation of H-2K^d Tetramers

The bacterial strain expressing the human β2m (hβ2m) was kindly provided by D. Garboczi. A DNA fragment coding for the BirA biotinylation substrate (5′-GGATCCCTGCATCATATTCTGGATGCACAGAA AATGGTGTGGAATCATCGTTAAAAGCTT-3′) was introduced into the bacterial expression vector pET-22b(+) (Novagen, Madison, WI) between two unique restriction sites BamHI and HindIII. The extracellular portion of the H-2K^d molecules (residues 1–280) was further introduced into this vector by PCR, using the following primers: 5′-GGGAATTCATATGGGCCCACATTCGCTGAGG-3′ and 5′- ACCC TGAGATGGAAGCTGCCTCCATCCACTGGATCCGCGCGCGGATCC AGTGGATGGAGGCAGCTTCCATCTCAGGGT-3′. After transformation into BL21(DE3)pLysS, complexes of K^d/hβ2m/peptide were produced as described in Garboczi et al. (1992) and purified by FPLC on Superdex 200 column (Pharmacia). Complexes (400 μg/ml) were further enzymatically biotinylated by incubation with 1 μg/ml of the BirA enzyme (Avidity, Denver, CO) for 14 hr at 25°C in the following buffer: Tris (pH 8), 20 mM; NaCl, 150 mM; $MgCl_2$, 5 mM; biotin, 200 μM; ATP, 5 mM; pepstatin, 1 μg/ml; leupeptin, 1 μg/ml; and phenylmethylsulfonylfluoride, 2 μM. After removal of free biotin by ultrafiltration on 10 kDa cut-off centrifugal filters (Millipore, Bedford, MA), biotinylated complexes were mixed with phycoerythrin-labeled UltraAvidin (Leinco Technologies, Ballwin, MI) at a 4 to 1 molar ratio.

Antibodies and Flow Cytometry

CyChrome-conjugated anti-CD8 (53-6.7) and FITC-conjugated anti-CD62L(Mel14) were purchased from Pharmingen (San Diego, CA); FITC-conjugated anti-CD8, biotinylated anti-B220, and anti-CD4 were purchased from Caltag (South San Francisco, CA). Spleen cells were depleted of B220$^+$ and CD4$^+$ cells using biotinylated MAbs and streptavidin beads (Dynals, Oslo, Norway). Cells were incubated for 2 hr at 4°C with PE-labeled MHC–peptide tetramers, washed, and incubated with the indicated antibodies. Cell sorting was performed on FACStar Plus (Becton Dickinson, San Jose, CA). Cell purity after sorting was analyzed by flow cytometry and varied from 87% to 99%.

RNA Extraction and cDNA Synthesis

Total RNA was isolated from cell samples using Trizol reagent (GIBCO BRL, Gaithersburg, MD), as recommended by the manufacturer, with the addition of 20 μg/ml of glycogen (Boehringer Mannheim, Manheim, Germany). cDNA was synthesized using $(dT)_{17}$ primer and M-MLV reverse transcriptase (GIBCO BRL) in the presence of RNasin (Promega, Madison, WI).

PCR Analyses

Immunoscope analysis, BC-, and TCRBV-specific primers have been previously described (Pannetier et al., 1993a). The CD3ε-specific primers were 5′-GCCTCAGAAGCATGATAAGC-3′ and 5′-FAM-CCCAGAGTGATACAGATGTC-3′. AV8-specific primer was described in Casanova et al. (1991). Semiquantitative PCR was essentially performed as in Azuara et al. (1997). Briefly, cDNA was prepared from total CD8$^+$ cells of a naive DBA/2 mouse or from the specific sorted T cell populations. For each of these samples, serial dilutions of cDNA were amplified by PCR (28 cycles) using a pair of CD3ε- or TCRBV-BC-specific primers. In each pair, the 3′ primer was fluorescent. PCR products were run on an automated sequencer. Fluorescent intensity was measured using the Immunoscope software. Ratio between TCRV and CD3ε was calculated for each sample. For each TCRV segment analyzed, ratio value obtained for the naive mouse was arbitrarily corrected to 1, and all the sample values were normalized on this basis.

Cloning of TCRβ Rearrangements

cDNA from immune mice were amplified by PCR using BV10- and BC-specific primers and cloned using a TA cloning kit (Invitrogen, The Netherlands). BV10-BJ1.2 rearrangements of naive mice displaying a 6 aa long CDR3β were cloned as follows. Spleens were prepared as single-cell suspensions and split into two samples. To

verify the reproducibility, the whole cloning and sequencing procedure was performed on these two samples for one of the mice. Splenocytes were depleted of $CD4^+$ cells. The cDNA was prepared and amplified by PCR using BV10- and BJ1.2-specific primers. After ethanol precipitation, PCR products were separated on an 8% polyacrylamide 7 M urea gel. Silver staining of the gel (DNA Silver Staining System, Promega) revealed five fragments spaced by three nucleotides and corresponding to in-frame transcripts. The band corresponding to the 6 aa long CDR3β was cut out from the gel and submitted to a second PCR of 20 cycles using the same primers. This final product was further purified on a 15% nondenaturing acrylamide gel and cloned using the Topo TA cloning kit (Invitrogen).

TCR Sequencing

PCR was carried out on *lacZ*$^-$ clones using RP and M13(-40) universal primers. This PCR product (3 μl) was incubated for 40 min at 37°C with 0.6 U of shrimp alkaline phosphatase (Amersham, United Kingdom) and 10 U of exonuclease (Amersham) in a total volume of 6 μl. After enzyme denaturation at 80°C for 20 min, sequencing reactions were performed using M13(-20) primer and the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA). Samples were run on a 373A or 377 DNA sequencer (Perkin-Elmer). Sequences were processed and analyzed using software designed for this purpose.

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References

Alt, F.W., Oltz, E.M., Young, F., Gorman, J., Taccioli, G., and Chen, J. (1992). VDJ recombination. Immunol. Today *13*, 306–314.

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

Azuara, V., Levraud, J.P., Lembezat, M.P., and Pereira, P. (1997). A novel subset of adult gamma delta thymocytes that secretes a distinct pattern of cytokines and expresses a very restricted T cell receptor repertoire. Eur. J. Immunol. *27*, 544–553.

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

Candeias, S., Waltzinger, C., Benoist, C., and Mathis, D. (1991). The $Vb17^+$ T cell repertoire: skewed Jβ usage after thymic selection; dissimilar CDR3s in $CD4^+$ versus $CD8^+$ cells. J. Exp. Med. *174*, 989–1000.

Casanova, J.-L., and Maryanski, J.L. (1993). Antigen-selected T-cell receptor diversity and self-nonself homology. Immunol. Today *14*, 391–394.

Casanova, J.-L., Romero, P., Widmann, C., Kourilsky, P., and Maryansky, J.L. (1991). T cell receptor genes in a series of class I major histocompatibility complex–restricted cytotoxic T lymphocyte clones specific for a plasmodium berghei nonapeptide: implications for T cell allelic exclusion and antigen-specific repertoire. J. Exp. Med. *174*, 1371–1383.

Casanova, J.-L., Cerottini, J.-C., Matthes, M., Necker, A., Gournier, H., Barra, C., Widmann, C., MacDonald, H.R., Lemonnier, F., Malissen, B., and Maryanski, J.L. (1992). H-2 restricted cytolytic T lymphocytes specific for HLA display T cell receptors of limited diversity. J. Exp. Med. *176*, 439–447.

Casanova, J.-L., Martinon, F., Gournier, H., Barra, C., Pannetier, C., Regnault, A., Kourilsky, P., Cerottini, J.-C., and Maryanski, J.L. (1993). T cell receptor selection by and recognition of two class I major histocompatibility complex–restricted antigenic peptides that differ at a single position. J. Exp. Med. *177*, 811–820.

Cibotti, R., Cabaniols, J.-P., Pannetier, C., Delarbre, C., Vergnon, I., Kanellopoulos, J.M., and Kourilsky, P. (1994). Public and private Vβ TCR repertoires against hen egg white lysozyme (HEL) in non-transgenic versus HEL transgenic mice. J. Exp. Med. *180*, 861–872.

Das, M.R., Cohen, A., Zamvil, S.S., Offner, H., and Kuchroo, V.K. (1996). Prior exposure to superantigen can inhibit or exacerbate autoimmune encephalomyelitis: T-cell repertoire engaged by the autoantigen determines clinical outcome. J. Neuroimmunol. *71*, 3–10.

Davis, M.M., and Bjorkman, P.J. (1988). T-cell antigen receptor genes and T-cell recognition. Nature *334*, 395–401.

Dembic, Z., Haas, W., Weiss, S., McCubrey, J., Kiefer, H., von Boehmer, H., and Steinmetz, M. (1986). Transfer of specificity by murine alpha and beta T-cell receptor genes. Nature *320*, 232–238.

Dudley, E.C, Petrie, H.T., Shah, L.M., Owen, M.J., and Hayday, A.C. (1994). T cell receptor β chain gene rearrangement and selection during thymocyte development in adult mice. Immunity *1*, 83–93.

Garboczi, D.N., Hung, D.T., and Wiley, D.C. (1992). HLA-A2-peptide complexes: refolding and crystallization of molecules expressed in Escherichia coli and complexed with single antigenic peptides. Proc. Natl. Acad. Sci. USA *89*, 3429–3433.

Kato, T., Suzuki, S., Sasakawa, H., Masuko, K., Ikeda, Y., Nishioka, K., and Yamamoto, K. (1994). Comparison of the J beta gene usage among different T cell receptor V beta families in spleens of C57BL/6 mice. Eur. J. Immunol. *24*, 2410–2414.

Kuida, K., Furutani, S.M., Saito, T., Kishimoto, H., Sano, K., and Tada, T. (1991). Post-translational attainment of allelic exclusion of the T cell receptor alpha chain in a T cell clone. Int. Immunol. *3*, 75–82.

Kumar, V., and Sercarz, E. (1994). Holes in the T cell repertoire to myelin basic protein owing to the absence of the D beta 2-J beta 2 gene cluster: implications for T cell receptor recognition and autoimmunity. J. Exp. Med. *179*, 1637–1643.

Levraud, J.-P., Pannetier, C., Langlade-Demoyen, P., Brichard, V., and Kourilsky, P. (1995). Recurrent TCR rearrangements in the CTL response in vivo against the P815 murine tumor. J. Exp. Med. *183*, 439–449.

Malissen, M., Trucy, J., Letourneur, F., Rebai, N., Dunn, D.E., Fitch, F.W., Hood, L., and Malissen, B. (1988). A T cell clone expresses two T cell receptor α genes but uses one αβ heterodimer for allorecognition and self-MHC-restricted antigen recognition. Cell *55*, 49–59.

Mallick, C.A., Dudley, E.C., Viney, J.L., Owen, M.J., and Hayday, A.C. (1993). Rearrangement and diversity of T cell receptor β chain genes in thymocytes: a critical role for the β chain in development. Cell *73*, 513–519.

Martinon, F., Cornille, F., Gomard, E., Fournie, Z.M., Abastado, J.P., Roques, B.P., and Levy, J.P. (1989). Two epitopes and one agretope map to a single HLA-A2 peptide recognized by H-2-restricted T cells. J. Immunol. *142*, 3489–3494.

Maryanski, J., Accolla, R., and Jordan, B. (1986). H-2-restricted recognition of cloned HLA class I gene products expressed in mouse cells. J. Immunol. *136*, 4340–4347.

Maryanski, J.L., Jongeneel, C.V., Bucher, P., Casanova, J.L., and Walker, P.R. (1996). Single-cell PCR analysis of TCR repertoires selected by antigen in vivo: a high magnitude CD8 response is comprised of very few clones. Immunity *4*, 47–55.

McHeyzer-Williams, M.G., and Davis, M.M. (1995). Antigen-specific development of primary and memory T cells in vivo. Science *268*, 106–111.

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

Nanda, N.K., Apple, R., and Sercarz, E. (1991). Limitations in plasticity of the T-cell receptor repertoire. Proc. Natl. Acad. Sci. USA *88*, 9503–9507.

Pannetier, C., Cochet, M., Darche, S., Casrouge, A., Zöller, M., and Kourilsky, P. (1993a). The sizes of the CDR3 hypervariable regions of the murine T cell receptor β chains vary as a function of the recombined germ-line segments. Proc. Natl. Acad. Sci. USA *90*, 4319–4323.

Pannetier, C., Delassus, S., Darche, S., Saucier, C., and Kourilsky, P. (1993b). Quantitative titration of nucleic acids by enzymatic amplification reactions run to saturation. Nucleic. Acids Res. *21*, 577–583.

Pannetier, C., Even, J., and Kourilsky, P. (1995). T-cell repertoire diversity and clonal expansions in normal and clinical samples. Immunol. Today *16*, 176–181.

Regnault, A., Levraud, J.P., Lim, A., Six, A. , Moreau, C., Cumano, A., and Kourilsky, P. (1996) The expansion and selection of T cell receptor alpha beta intestinal intraepithelial T cell clones. Eur. J. Immunol. *26*, 914–921.

Saito, T., Sussman, J.L., Ashwell, J.D., and Germain, R.N. (1989). Marked differences in the efficiency of expression of distinct alpha beta T cell receptor heterodimers. J. Immunol. *143*, 3379–3384.

Sant'Angelo, D.B., Waterbury, P.G., Cohen, B.E., Martin, W.D., Van, K.L., Hayday, A.C., and Janeway, C.J. (1997). The imprint of intrathymic self-peptides on the mature T cell receptor repertoire. Immunity *7*, 517–524.

van Meerwijk,. J.P.M., Marguerat, S., Lees, R.K., Germain, R.N., Fowlkes, B.J., and MacDonald, H.R. (1997). Quantitative impact of thymic clonal deletion on the T cell repertoire. J. Exp. Med. *185*, 377–383.

van Pel, A., De Plaen, E., and Boon, T. (1985). Selection of highly transfectable variant from mouse mastocytoma P815. Somat. Cell Mol. Genet. *11*, 467–475.