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# A Kinetic Basis For T Cell Receptor Repertoire Selection during an Immune Response

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## Summary

**The basis for T cell antigen receptor (TCR) repertoire selection upon repeated antigenic challenge is unclear. We evaluated the avidity and dissociation kinetics of peptide/major histocompatibility complex (MHC) tetramer binding to antigen-specific T lymphocytes isolated following primary or secondary immunization. The data reveal a narrowing of the secondary repertoire relative to the primary repertoire, largely resulting from the loss of cells expressing TCRs with the fastest dissociation rates for peptide/MHC binding. In addition, T cells in the secondary response express TCRs of higher average affinity for peptide/MHC than cells in the primary response. These results provide a link between the kinetics and affinity of TCR-peptide/MHC interactions and TCR sequence selection during the course of an immune response.**

## Introduction

In most cases, antigenic challenge results in the establishment of immunological memory, a state in which the immune system is poised to respond more rapidly and effectively upon recurrent antigenic exposure. Recall responses are often associated with a refinement of antigen-specific immunoglobulin and T cell antigen receptor (TCR) repertoires. Immunoglobulin molecules function by directly binding antigen, thereby linking immune effector mechanisms to specific antigens. Upon repeated antigenic challenge, affinity maturation improves the efficiency of this linkage by selecting for immunoglobulins of increased affinity for antigen. αβ T lymphocytes, on the other hand, recognize antigenic peptides presented by major histocompatibility complex (MHC) molecules on the surface of antigen-presenting cells (APCs). Antigen recognition is mediated by the TCR αβ heterodimer. Effective APC-mediated stimulation triggers context-dependent T cell developmental or effector processes. In contrast to immunoglobulins, the basis for TCR repertoire selection and the consequences of repertoire changes on T cell function are not well understood.

In the cytochrome c model antigen system, immunization of H-2$^k$ mice with cytochrome c protein in adjuvant primes a specific CD4$^+$ T helper response primarily directed toward an immunodominant epitope restricted by the I-E$^k$ MHC molecule (Schwartz, 1985). McHeyzer-Williams and Davis (1995) have shown that primary T cells responding to cytochrome c exhibit a broader range of α and β chain TCR sequences than do secondary cells, suggesting that particular TCR heterodimers are selected during the course of the response. What is the biochemical basis of this selection? Several studies (Matsui et al., 1994; Alam et al., 1996; Lyons et al., 1996; Kersh et al., 1998b) have demonstrated that the biological effect of a peptide/MHC-TCR interaction often correlates with the half-life ($t_{1/2}$) of the interaction, suggesting that the duration of the TCR-ligand interaction is a primary determinant of ligand activity. Importantly, Kersh et al. (1998b) have also noted that peptide/MHC ligands with similar affinities for TCR can differ drastically with respect to their potency for T cell activation, suggesting that the affinity of a TCR-ligand interaction is a poor predictor of ligand potency.

Recently, the development of antigen-specific T cell staining approaches based on tetrameric (Altman et al., 1996) or dimeric (Greten et al., 1998) peptide/MHC complexes has made it possible to directly identify populations of antigen-specific T cells. Here, we have extended the use of multimeric peptide/MHC reagents to evaluate the ligand-binding properties of antigen-specific cells en masse. We evaluated the avidity and dissociation kinetics of peptide/MHC tetramer binding to antigen-specific T cells that were isolated following primary or secondary immunization. Data from tetramer-based assays were utilized to estimate relative differences between primary and secondary populations with respect to the ligand-binding characteristics of expressed TCRs. Results provide a link between the kinetics and affinity of TCR-peptide/MHC interactions and TCR sequence selection during an immune response.

## Results

### Kinetics of Tetramer Staining Decay

Previously, it has been shown that the level of peptide/MHC tetramer binding to peptide-specific hybridoma cells correlates with the affinity of TCR for peptide/MHC (Crawford et al., 1998). In order to determine whether peptide/MHC tetramers could be used for kinetic determinations as well, we made I-E$^k$ tetramers complexed with either the moth cytochrome c 88–103 (MCC) peptide or the variant peptide MCC(102S) (Boniface et al., 1998; Gütgemann et al., 1998). Previous studies using surface plasmon resonance (Matsui et al., 1994; Lyons et al., 1996) have shown that the 2B4 TCR αβ heterodimer binds to MCC/I-E$^k$ with a half-life ($t_{1/2}$) of 11 s and MCC(102S)/I-E$^k$ with a $t_{1/2}$ of 2 s at 25°C (Table 1). Recent work has also indicated that the 5C.C7 TCR has a $t_{1/2}$ for MCC/I-E$^k$ binding between those of the 2B4-MCC/I-E$^k$ and 2B4-MCC(102S)/I-E$^k$ interactions (Lyons, D.S., Teyton, L., and Davis, M.M., unpublished data). We stained T cells from mice transgenic for either the 2B4

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Table 1. Summary of MCC/I-E$^k$ Tetramer Staining $t_{1/2}$ Values

| Cells | Tetramer | N | Time Interval (min) | Mean Slope[a] ($min^{-1}$) | $t_{1/2}$[b] (min) | Surface Plasmon Resonance 25°C: Monomer $t_{1/2}$[c] (s) | Surface Plasmon Resonance 25°C: Tetramer $t_{1/2}$[d] (min) |
|---|---|---|---|---|---|---|---|
| Primary | MCC/I-E$^k$ | 20 | 0–20 | 0.0271 | 26 | | |
| | | | 20–40 | 0.0148 | 47 | | |
| | | | 40–60 | 0.0164 | 42 | | |
| | | | 60–90 | 0.0064 | 110 | | |
| | | | 90–120 | 0.0052 | 130 | | |
| | | | 0–120 | 0.0126 | 55 | | |
| Secondary | MCC/I-E$^k$ | 19 | 0–20 | 0.0178 | 39 | | |
| | | | 20–40 | 0.0157 | 44 | | |
| | | | 40–60 | 0.0136 | 51 | | |
| | | | 60–90 | 0.0080 | 86 | | |
| | | | 90–120 | 0.0039 | 180 | | |
| | | | 0–120 | 0.0109 | 64 | | |
| 5C.C7 αβ | MCC/I-E$^k$ | 6 | | | 21 ± 2 | | |
| 5C.C7 β | MCC/I-E$^k$ | 2 | | | 61 ± 5 | | |
| 2B4 αβ | MCC/I-E$^k$ | 3 | | | 240 ± 30 | 11.0 | 32.0 |
| 2B4 αβ | MCC(102S)/I-E$^k$ | 4 | | | 14 ± 3 | 2.0 | 2.4 |

[a] The slope is equivalent to $\ln(F_a/F_b)/t$, where $F_a$ is the normalized fluorescence at the start of the interval, $F_b$ is the normalized fluorescence at the end of the interval, and t is the length of the interval (minutes). Unlike Figure 2B, time is expressed in minutes instead of hours to facilitate the determination of $t_{1/2}$, which is best expressed in minutes.
[b] $t_{1/2}$ is equal to ln 2 divided by the mean slope.
[c] Data taken from Lyons et al. (1996).
[d] Data taken from Boniface et al. (1998).

or 5C.C7 TCR with MCC/I-E$^k$ tetramer for 45 min at room temperature, washed the cells, cooled them to 4°C, and then measured the rate of decay using flow cytometry. To prevent rebinding of MCC/I-E$^k$ moieties during the tetramer dissociation assay, we incubated samples with saturating amounts of the 14.4.4 monoclonal antibody, which binds I-E$^k$ regardless of which peptide is bound. As shown in Figure 1, in each case we obtained linear decay plots of the natural logarithm of the normalized fluorescence versus time, indicating that tetramer decay

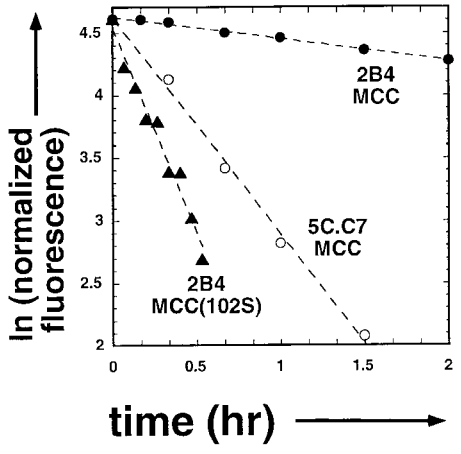


Figure 1. Tetramer Staining Decay Kinetics for 2B4 or 5C.C7 αβ TCR Transgenic Cells Stained with Peptide/I-E$^k$ Tetramer

Representative decay plots of the natural logarithm of the normalized fluorescence versus time after 14.4.4 addition for 2B4 cells stained with MCC/I-E$^k$ tetramer (closed circles), 2B4 cells stained with MCC(102S)/I-E$^k$ tetramer (closed triangles), or 5C.C7 cells stained with MCC/I-E$^k$ tetramer (open circles).

was occurring stochastically and that the resulting tetramer staining half-lives should be proportional to the half-lives of respective TCR-peptide/MHC complexes. Consistent with this, we found that the MCC/I-E$^k$ tetramer label on 2B4 transgenic cells decayed with a $t_{1/2}$ of approximately 240 min, while the MCC(102S)/I-E$^k$ tetramer decayed at a much faster rate, with a $t_{1/2}$ of ~14 min (Figure 1 and Table 1). The fact that this represents a ~20-fold decrease in $t_{1/2}$ versus an approximately 6-fold decrease in $t_{1/2}$ for the respective bimolecular interactions likely reflects the multivalency of tetramer binding. The ~20-fold difference is also consistent with the surface plasmon resonance studies of Boniface et al. (1998), which revealed a ~13-fold difference in the $t_{1/2}$ of MCC/I-E$^k$ tetramer and MCC(102S)/I-E$^k$ tetramer binding to immobilized 2B4 TCR at 25°C (32.0 min and 2.4 min, respectively [Table 1]). As an additional comparison, we found that MCC/I-E$^k$ tetramer binding decayed from 5C.C7 T cells with a $t_{1/2}$ of ~20 min (Figure 1 and Table 1). The 5C.C7 TCR heterodimer is typical of both cytochrome c–reactive hybridomas (Hedrick et al., 1988; Jorgensen et al., 1992) and cytochrome c–specific single cells isolated following immunization (McHeyzer-Williams and Davis, 1995) with respect to CDR3 sequences, whereas the 2B4 CDR3 sequences are unique.

To evaluate TCR repertoire selection with respect to TCR-peptide/MHC dissociation, we analyzed cytochrome c–specific cells isolated after either primary or secondary immunization (Figure 2). Lymphocytes were enriched for CD4$^+$ cells, stained with MCC/I-E$^k$ tetramer, and incubated with 14.4.4. At appropriate time points, aliquots were removed and analyzed via flow cytometry. For each sample, the natural logarithm of the normalized fluorescence was plotted versus time after 14.4.4 addition (Figure 3A). As a reference, representative decay plots obtained previously for 2B4 T cells stained with either

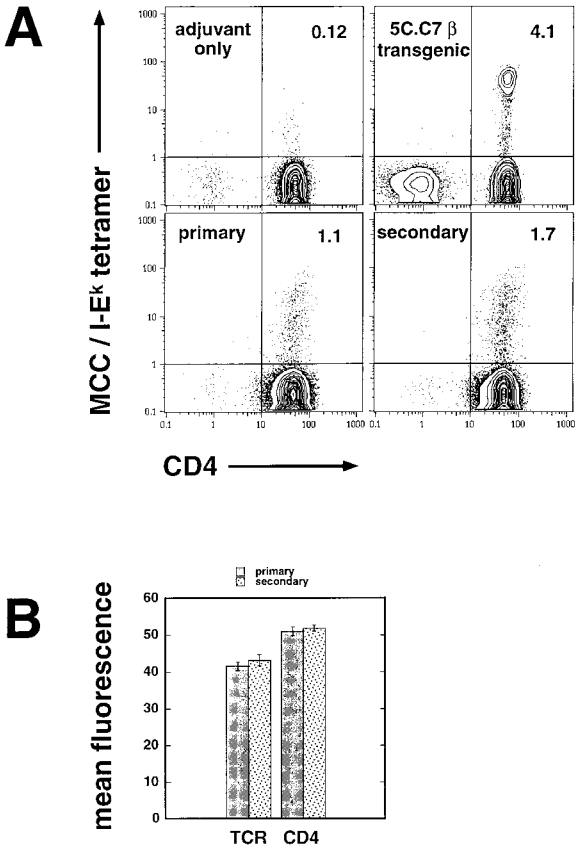


Figure 2. Flow Cytometric Analysis of Cytochrome c–Specific T Cells Using MCC/I-E$^k$ Tetramer

(A) Representative probability plots of MCC/I-E$^k$ tetramer staining and CD4 expression are depicted for the indicated populations. Adjuvant only, primary, and secondary refer to populations isolated following immunization. Cells from a mouse transgenic for the β chain of the 5C.C7 TCR were stained as a positive control. The percentage of displayed cells falling within the upper right quadrant is indicated. See Experimental Procedures for details.

(B) Comparison of TCR and CD4 expression levels for cells isolated after primary or secondary immunization. Bars represent mean fluorescence ± SEM. Data are from representative experiments (n = 10 for each group in TCR comparison, n = 20 for each group in CD4 comparison).

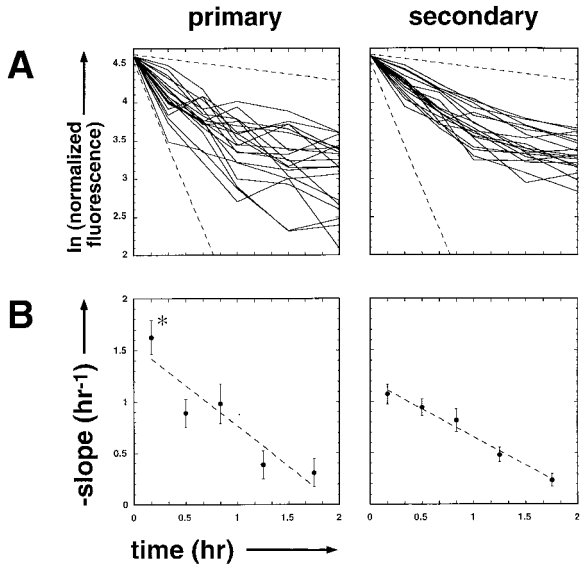


Figure 3. Tetramer Staining Decay Kinetics for Primary or Secondary Populations Stained with MCC/I-E$^k$ Tetramer

(A) Staining decay plot overlays for 20 primary and 19 secondary samples. The natural logarithm of the normalized fluorescence is plotted versus time after 14.4.4 addition. Tetramer staining was evaluated at 0, 20, 40, 60, 90, 120, and 180 min after 14.4.4 addition. Mean decay rates for primary and secondary populations were comparable over the 120–180 min interval (data not shown). The upper and lower dashed lines represent MCC/I-E$^k$ tetramer dissociation and MCC(102S)/I-E$^k$ tetramer dissociation from 2B4 cells, respectively.

(B) Quantitative analysis of tetramer staining decay kinetics. For each time interval of the plots depicted in (A), the mean slope (± SEM) of an interval is plotted versus the midpoint of that interval for primary and secondary groups. The slope is equivalent to $\ln(F_a/F_b)/t$, where $F_a$ is the normalized fluorescence at the start of the interval, $F_b$ is the normalized fluorescence at the end of the interval, and t is the length of the interval (hours). The asterisk indicates a statistically significant difference ($p < 0.01$) between primary and secondary groups over the 0–20 min interval.

MCC/I-E$^k$ tetramer or MCC(102S)/I-E$^k$ tetramer are shown as dashed lines (Figure 3A). These data revealed significant animal-to-animal variation among primary populations yet minimal variation among secondary populations. This suggests that while the composition of primary cytochrome c–reactive populations generally varies with respect to dissociation rate ($k_{off}$), secondary immunization consistently selects for an antigen-specific population of a more restricted, defined composition. A pairwise comparison of the decay kinetics over each time interval (Figure 3B) revealed that the relatively fast decay component (mean population $t_{1/2}$ of ~25 min) prominent in the 0–20 min time interval of the majority of primary populations is generally absent among secondary populations ($p < 0.01$). This comparison suggests that a proportion of primary cells expressing TCRs with the highest dissociation rates for peptide/MHC binding are selected against prior to day 4 of the secondary response. Over later time intervals (>20 min), primary decay curves were generally comparable to those of secondary populations (Figure 3A), suggesting that primary and secondary repertoires overlap considerably. These results are consistent with the findings of McHeyzer-Williams and Davis (1995), which reveal a strong selection for canonical CDR3 junctional motifs in the primary response (with ~70% of antigen-specific cells expressing canonical TCRs) yet further selection for canonical motifs in the secondary response (>90% of antigen-specific cells). Thus, while primary and secondary repertoires overlap considerably, continued narrowing of the secondary repertoire relative to the primary repertoire takes place, largely resulting from the loss of cells expressing TCRs with the highest dissociation rates for peptide/MHC binding.

For each time interval, the average of the mean population decay rates and corresponding estimated half-lives for populations isolated after primary and secondary immunization are summarized in Table 1. Throughout the duration of the analysis, for both primary and secondary groups, estimated half-lives ranged between 25 and 200 min. Using our calibration studies of MCC/I-E$^k$ tetramer and MCC(102S)/I-E$^k$ tetramer decay from 2B4 cells ($t_{1/2}$ ~240 min and ~14 min, respectively) as benchmarks, we conclude that the majority of MCC-specific

cells in primary and secondary T cell populations bear TCRs that bind MCC/I-E$^k$ with a $t_{1/2}$ between 2 and 11 s.

Several lines of evidence suggest that differential expression of the CD4 coreceptor is unlikely to account for observed tetramer dissociation differences. First, CD4 expression levels were found to be comparable for cells isolated following primary or secondary immunization in all experiments (Figure 2B). Second, tetramer staining was performed concurrently with staining using the GK1.5 anti-CD4 antibody. GK1.5 treatment has been shown to diminish T cell responsiveness to tetramer stimulation, most likely by blocking CD4-MHC class II interactions (Boniface et al., 1998). Third, Hamad et al. (1998) have demonstrated that dimeric peptide/MHC complexes dissociate from peptide-specific hybridoma cells at a rate that is independent of CD4 expression, indicating that CD4 is unlikely to influence peptide/MHC multimer dissociation. Lastly, Crawford et al. (1998) found essentially no difference in tetramer binding between CD4$^+$ and CD4$^-$ T cell hybridomas.

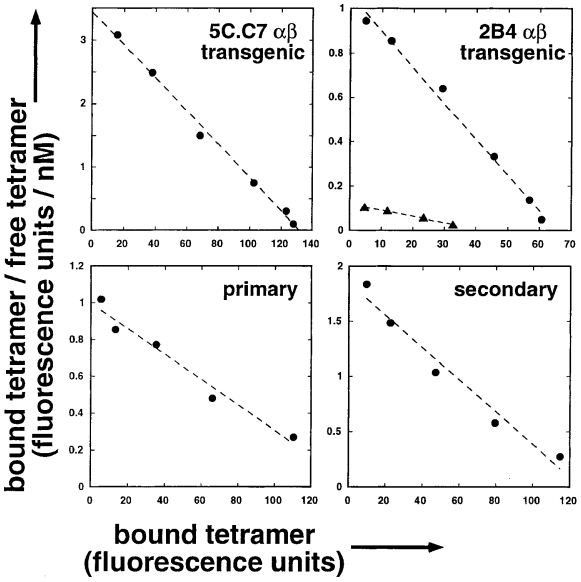


Figure 4. Scatchard Analysis of Peptide/I-E$^k$ Tetramer Binding to Cytochrome c–Specific T Cells

The indicated representative T cell population was stained with MCC/I-E$^k$ tetramer (closed circles) or MCC(102S)/I-E$^k$ tetramer (closed triangles) over a range of concentrations. Apparent $K_D$ values were derived from Scatchard plots of bound tetramer/free tetramer versus bound tetramer. See Experimental Procedures for details.

### Extent of Tetramer Binding under Equilibrium Staining Conditions

In order to compare primary and secondary cells with respect to the affinity of expressed TCRs for MCC/I-E$^k$, tetramer binding was evaluated under equilibrium staining conditions. MCC/I-E$^k$ tetramer staining of cytochrome c–specific cells was found to reach equilibrium within 2 hr when staining was performed at room temperature (data not shown). Preliminary studies revealed a modest but statistically significant increase in tetramer staining intensity of cells isolated following secondary immunization in comparison to cells isolated following primary immunization (data not shown). Potential factors influencing the tetramer staining intensity of antigen-specific cells include TCR expression level, the affinity of TCR for peptide/MHC, and the expression level of the CD4 coreceptor. Two findings suggest that the observed differences in tetramer staining intensity reflect differences in TCR affinity. First, as discussed previously, neither Crawford et al. (1998) nor Hamad et al. (1998) found an influence of CD4 expression on multimer staining. Second, in our experiments, TCR and CD4 levels were comparable for cells isolated following primary or secondary immunization (Figure 2B). Thus, the observed differences in staining intensity were likely due to differences in TCR affinity for peptide/MHC.

We determined apparent $K_D$ values for tetramer binding to cytochrome c–specific cells isolated following primary or secondary immunization by Scatchard analysis (Figures 4 and 5). In general, MCC/I-E$^k$ tetramer binding to cells of a secondary response exhibited a lower apparent $K_D$ (79 $\pm$ 13 nM) than for binding to cells of a primary response (120 $\pm$ 40 nM). This difference in apparent $K_D$ is statistically significant, with a p value of $<$0.002. This indicates that the MCC/I-E$^k$ tetramer binds cells of a secondary response with a higher average avidity than cells of a primary response and implies that cells isolated following secondary immunization express TCRs of somewhat higher average affinity for peptide/MHC ligand relative to antigen-specific cells of a primary response. As $K_D = k_{off}/k_{on}$ ($k_{on}$ = association rate), this effect may be due entirely to the elimination of the population of T cells expressing TCRs with the fastest dissociation rates. However, it is also possible that differences in TCR-peptide/MHC association rate may contribute to observed differences in apparent $K_D$.

For comparison, apparent $K_D$ values were determined for the binding of tetramer to cytochrome c–specific transgenic T cells (Figures 4 and 5). MCC/I-E$^k$ tetramer bound to 2B4 transgenic cells with an apparent $K_D$ of 60 $\pm$ 9 nM, an avidity comparable to that of binding to cells isolated following secondary immunization ($K_D \sim$80 nM). Thus, we estimate that cells of a secondary response, on average, express TCRs with an affinity for MCC/I-E$^k$ comparable to that of the 2B4 TCR, estimated to be between 40–90 $\mu$M at 25°C (Matsui et al., 1994; Lyons et al., 1996). In contrast to the MCC/I-E$^k$ tetramer, tetramers made with the weak agonist MCC(102S) bound 2B4 cells with approximately 5-fold lower avidity ($K_D$ of 260 $\pm$ 110 nM).

Interestingly, the MCC/I-E$^k$ tetramer was found to bind 5C.C7 $\alpha\beta$ transgenic cells with a lower apparent $K_D$ (34 $\pm$ 7 nM) relative to 2B4 binding, despite having a faster dissociation rate (Figure 1 and Table 1). Thus, 5C.C7 is likely to have a faster association rate for MCC/I-E$^k$ relative to 2B4. What could account for the putative difference in association rate? In some cases, electrostatic forces have been shown to increase the rate of protein association (Schreiber and Fersht, 1996). A more rapid association rate for 5C.C7 relative to 2B4 may be due to the formation of a salt bridge between a 5C.C7 $\alpha$ chain glutamate residue and a lysine of the MCC peptide (Jorgensen, et al., 1992). The 2B4 $\alpha$ chain lacks the crucial glutamate residue at that position and thus could not form an equivalent salt bridge with the MCC peptide.

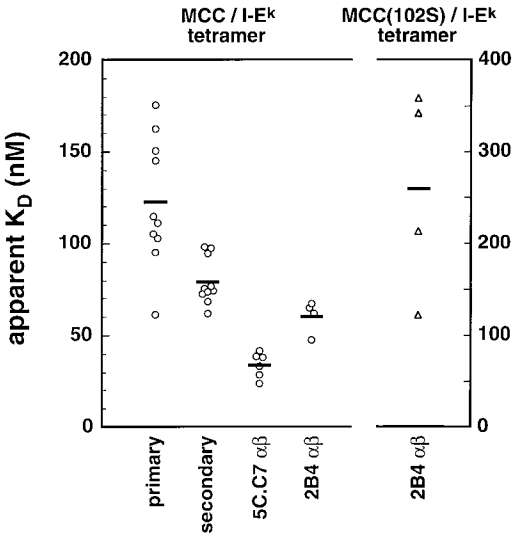


Figure 5. Summary Plot of Apparent $K_D$ Values for Peptide/I-$E^k$ Tetramer Binding to Cytochrome c–Specific T Cells

Apparent $K_D$ values for MCC/I-$E^k$ tetramer (open circles) or MCC(102S)/I-$E^k$ tetramer (open triangles) binding to cytochrome c–specific populations of individual mice of the indicated type (bottom) are presented. Horizontal bars indicate mean apparent $K_D$ values. The observed difference between primary and secondary populations is statistically significant ($p < 0.002$). Left axis labels indicate the scale for MCC/I-$E^k$ tetramer staining, the right axis labels for MCC(102S)/I-$E^k$ tetramer staining. MCC(102S)/I-$E^k$ tetramer binds 5C.C7 αβ T cells with too low of an avidity to accurately determine apparent $K_D$ values.

Due to the low avidity of MCC(102S)/I-$E^k$ tetramer binding to 2B4 cells, Scatchard plots yielded slopes that were significantly smaller than slopes for other $K_D$ determinations. Since experimental errors/variations affect Scatchard plots with small slopes to a greater extent than those with larger slopes, apparent $K_D$ determinations for MCC(102S)/I-$E^k$ tetramer binding to 2B4 cells showed increased variability relative to other determinations.

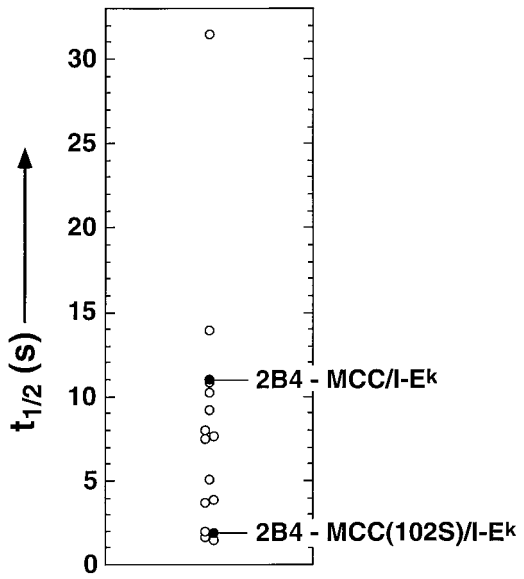


Figure 6. Scatter Plot Summarizing $t_{1/2}$ Values for Agonist or Weak Agonist Peptide/MHC Class II Interactions with Specific TCR

All measurements were made at 25°C using surface plasmon resonance. Half-lives for the interaction of 2B4 with MCC/I-$E^k$ and MCC(102S)/I-$E^k$ are indicated. Data are taken from Matsui et al., 1994; Lyons et al., 1996; Khandekar et al., 1997; Seibel et al., 1997; Crawford et al., 1998; Liu et al., 1998; and Kersh et al., 1998b.

## Discussion

Comparison of primary and secondary effector populations with respect to the kinetics of peptide/MHC tetramer dissociation reveals selection against a proportion of cells expressing TCRs with the highest dissociation rates for peptide/MHC binding. This observation implies that TCR repertoire selection during an immune response is likely based on a selection for cells within the peripheral repertoire expressing TCRs that bind peptide/MHC for the longest duration. For antigen-specific T cells isolated following immunization with cytochrome c, the optimal $t_{1/2}$ appears to lie somewhere between 2–11 s (at 25°C), a range that is consistent with previously published TCR-peptide/MHC class II interactions of various specificities (Figure 6). The finding that a narrow primary $t_{1/2}$ distribution ($2 < t_{1/2} < 11$ s) is narrowed further in the secondary response suggests that T cell selection during an immune response is sensitive to subtle differences in the duration of TCR-ligand interactions. These findings are perhaps best interpreted in the context of a kinetic model of T cell signaling.

In a kinetic model, the biological implications of a TCR-peptide/MHC interaction are primarily determined by the duration of the interaction (Matsui et al., 1994; McKeithan, 1995; Lyons et al., 1996; Rabinowitz et al., 1996). In addition, the process of kinetic proofreading has been proposed to account for the high sensitivity and high selectivity of T cell antigen recognition (Rabinowitz et al., 1996). Kinetic proofreading is based on a time delay between antigen binding and receptor signaling events that is dictated by the completion of multiple intermediate steps. Kinetic models are supported by several studies correlating the duration of a peptide/MHC-TCR interaction with biological events associated with successful signaling. First, agonist peptide/MHC complexes generally have longer half-lives for TCR binding relative to antagonist peptide/MHC complexes (Matsui et al., 1994; Lyons et al., 1996; Kersh et al., 1998b). Second, the half-life of a peptide/MHC-TCR interaction correlates with the induction of TCR CD3-ζ phosphorylation patterns associated with T cell stimulation (Kersh et al., 1998a, 1998b). Third, the half-life of peptide/MHC binding to TCR correlates with the capacity to induce TCR or peptide/MHC clustering at the APC-T cell interface (Monks et al., 1998; A. Grakoui et al., unpublished data). In particular, Grakoui and colleagues have monitored the distribution of peptide/MHC molecules within lipid bilayers at T cell-bilayer interfaces and found a correlation between the size and density of peptide/MHC clusters and the $t_{1/2}$ of a TCR-peptide/MHC interaction.

TCR cluster formation has been proposed (McKeithan, 1995; Davis et al., 1998) as an important intermediate in a kinetic proofreading model of T cell signaling. Reich et al. (1997) have demonstrated that in solution, specific peptide/MHC-TCR complexes oligomerize at concentrations near the $K_D$ of the binding reaction, suggesting that successful TCR-peptide/MHC ternary complexes have an inherent affinity for each other. In addition, studies of Boniface et al. (1998) employing peptide/

MHC tetramers to stimulate T cells suggest that multiple TCR complexes must be brought together to trigger early intracellular signals.

Placing the observations presented here in the context of the kinetic signaling and TCR clustering models discussed above, we propose that during an ongoing immune response and/or upon successive antigenic exposure, antigen-specific cells expressing TCRs that bind peptide/MHC ligand for a longer duration are selectively preserved and/or expanded based on an optimal capacity to cluster TCRs and initiate signaling cascades. For peptide/MHC-TCR interactions of shorter duration, ternary complexes may exist too transiently to allow extensive self-aggregation, thereby limiting TCR cluster formation. During antigen recognition, the process of TCR cluster formation may contribute to the amplification of subtle differences in the duration of TCR-ligand interactions into larger differences in T cell responsiveness or function.

Since T cells lack the somatic hypermutation mechanism which allows immunoglobulins to achieve very high affinities, TCR repertoire selection in the periphery is likely to occur by (1) selective T cell preservation during the contraction of the primary effector phase, (2) selective T cell survival during the resting memory phase, (3) selective T cell expansion upon secondary antigenic challenge, or (4) a combination of these mechanisms. The studies of Pamer and colleagues (Busch et al., 1998) are interesting in this regard. Using a panel of anti-TCR Vβ antibodies to evaluate antigen-specific TCR repertoire diversity in response to *Listeria monocytogenes*, they found that while the TCR repertoire of the memory pool reflects that of the primary effector pool, selective expansion of T cells bearing specific TCR Vβs is evident following secondary antigenic exposure. Thus, during the immune response to *Listeria*, post-primary TCR repertoire selection likely occurs following secondary antigenic challenge, suggesting that selective T cell expansion is the most likely mechanism of repertoire selection.

The serial engagement model of Lanzevecchia and colleagues (Valitutti et al., 1995; Valitutti and Lanzavecchia, 1997) proposes that a limited number (~100) of specific peptide/MHC complexes on the surface of an APC is capable of mediating the specific downregulation of thousands of TCRs on a T cell by transient, serial binding events. They postulate that a peptide/MHC complex must engage a TCR with sufficient duration to allow downregulation of the TCR and then dissociate, thereby becoming available to trigger subsequent TCRs. One prediction of this model would be that the distribution of half-lives of expressed TCR-peptide/MHC interactions for an antigen-specific T cell population would resemble a normal distribution centered around an optimum value. Repertoire selection during an ongoing immune response or upon secondary antigenic exposure would be expected to narrow this distribution while maintaining the optimal mean value. The experiments documented in this study demonstrate selection against a proportion of antigen-specific cells expressing TCRs of highest dissociation rate. However, selection against cells expressing TCRs of lowest dissociation rate was not evident. This may reflect limitations in our assay or may accurately reflect repertoire narrowing occurring between the primary and secondary effector phases of an immune response.

Analysis of primary responses with respect to TCR dissociation rate reveals significant animal-to-animal variation in TCR repertoire composition. Such variation is consistent with the findings of Bousso and colleagues (1998), who demonstrated that primary antigen-specific repertoires of individual mice vary with respect to TCR sequence and that these differences reflect differences in naive repertoires. Despite variations in primary repertoires, our results demonstrate that secondary antigenic challenge generally elicits an antigen-specific repertoire of limited complexity with respect to TCR dissociation rate.

The studies presented here provide evidence of qualitative differences between primary effector cells and secondary (memory) effectors with respect to the affinity and kinetics of expressed TCR binding to peptide/MHC ligand. This qualitative effect may contribute to the efficacy of T cell memory. For example, secondary responder pools enriched for cells that recognize peptide/MHC with an optimal kinetics should have an enhanced proliferative and effector capacity, thereby enhancing protective recall immunity.

Recently, Busch and Pamer (1999) have evaluated changes in peptide/MHC tetramer binding to antigen-specific T cells in a murine infectious disease model. They also observed a trend toward stronger binding and slower dissociation in a recall response, consistent with the data presented here.

## Experimental Procedures

### Mice and Immunization

B10.BR mice were purchased from the Jackson Laboratory. 5C.C7 TCR β chain and TCR αβ chain transgenic mice (Fazekas de St. Groth et al., 1992; Jorgensen et al., 1992) and 2B4 TCR αβ transgenic mice (Berg et al., 1989), all on the B10.BR background, were bred and maintained in the Research Animal Facility at Stanford University. Female mice were immunized with 2 mg/mL pigeon cytochrome c protein (Sigma) in Ribi MPL + TDM adjuvant (Ribi ImmunoChem Research) or adjuvant alone, 100 μL on each side of the base of the tail, using a syringe fitted with a 23G needle. To monitor primary responses or responses to adjuvant alone, B10.BR mice were immunized at >14 weeks of age and sacrificed at day 6 post-challenge. To monitor secondary responses, mice were immunized once at approximately 6 weeks of age, again at >14 weeks of age, and sacrificed at day 4 post-secondary challenge. This ensured that primary and secondary mice were age matched at the time of analysis. For analysis, cells from inguinal and periaortic lymph nodes were harvested and washed. Adjuvant only, primary, and secondary samples were enriched for $CD4^+$ cells using Dynabeads mouse CD4 in conjunction with DETACHaBEAD mouse CD4 (Dynal A. S., Oslo, Norway) using protocols recommended by the manufacturer.

### Flow Cytometry

Phycoerythrin (PE)-labeled I-$E^k$ tetramers bearing the wild-type moth cytochrome c 88–103 peptide (MCC) or the MCC variant MCC(102S) were prepared as described (Gütgemann et al., 1998). Likewise, a "dump" tetramer consisting of streptavidin-CyChrome (Pharmingen) multimerized I-$E^k$ bearing the null 93E,99T,102A variant of MCC 88–103 (Reay et al., 1994; Wülfing et al., 1997) was also prepared. All stains and washes were prepared using wash media consisting of 2% FCS, 0.075% sodium bicarbonate, and 0.1% sodium azide in modified RPMI-1640 (Sigma). For the analysis of tetramer staining at equilibrium, lymphocytes were stained for 3 hr at ambient temperature (22°C) with concentrations of tetramer ranging from 5 to 410 nM. Cells were also stained with fluorescein-labeled H57–597

(anti-mouse TCR Cβ, Pharmingen), CyChrome-labeled RA3–6B2 (anti-mouse CD45R/B220, Pharmingen), CyChrome-labeled 53–6.7 (anti-mouse CD8, Pharmingen), and CyChrome-labeled dump tetramer. Stained cells were washed three times, resuspended in wash media containing 1 μg/mL propidium iodide (PI), and analyzed on a Becton Dickinson FACScan flow cytometer. For the analysis of tetramer staining decay, cells were stained for 45 min at room temperature with the aforementioned peptide/I-$E^k$ tetramer-PE (135 nM), dump tetramer-CyChrome, anti-CD45R-CyChrome, and anti-CD8-CyChrome reagents in conjunction with fluorescein-labeled anti-mouse CD4 (GK1.5). Cells were washed three times and resuspended in 100 μL wash media containing 1 μg/mL PI and 100 μg/mL 14.4.4, an anti-I-$E^k$ monoclonal antibody. At appropriate time points, cells were resuspended, removed, and analyzed on a Becton Dickinson FACScan flow cytometer. For all analyses, >50,000 event files were collected and analyzed using FlowJo software (Stanford University). Cells positive for B220, CD8, propidium iodide, and dump tetramer staining, as well as cells of low side scatter, were gated out of the analysis.

**Flow Cytometric Data Analysis**

Complications in tetramer staining decay analyses stemmed from the use of the same reagent (MCC/I-$E^k$ tetramer) for both the identification of antigen-specific cells and the evaluation of tetramer binding. For example, in staining decay assays, as tetramer staining intensity decreases over time due to tetramer dissociation, the ability to detect a cell may be lost if its fluorescence intensity drops below the gate established to identify specific cells. Thus, over time, a large proportion of the sample population is "lost" due to an inability to detect it. For this reason, the use of the mean fluorescence intensity parameter is inappropriate. Instead, the parameter of "total fluorescence" within the tetramer$^+$ gate was deemed the most suitable parameter for tetramer staining decay analysis. The total fluorescence is the sum of the fluorescence intensities of MCC/I-$E^k$ tetramer-positive cells normalized per CD4 cell. The antigen-specific total fluorescence was determined by subtracting total fluorescence levels found in adjuvant only controls from the observed total fluorescence of a population isolated following immunization. This adjusted value was then normalized to percent of the total fluorescence at the initial time point and plotted on a logarithmic scale. For simplicity, total fluorescence values are listed as "fluorescence" in the figures. As an analogy to BIAcore (Pharmacia Biosensor) analysis, the composite cell surfaces of all cells of a tetramer$^+$ population can be thought of as the equivalent of a BIAcore surface coated with TCR. A measure of the total fluorescence of a tetramer-stained T cell sample is analogous to a measure of resonance units upon tetramer binding to a TCR-coated BIAcore surface.

**Scatchard Analysis**

Apparent $K_D$ values were derived from the negative reciprocal of the slope of the line fit to Scatchard plots of bound tetramer/free tetramer (fluorescence units/nM) versus bound tetramer (fluorescence units). Median population tetramer staining intensity was used as a measure of bound tetramer. Since tetramer was in vast excess even at the lowest stain concentration, the concentration of free tetramer simply reflects staining concentration.

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