

A New Look at T Cells

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Recent technological advances have shed unexpected light on the CD8⁺ T cell responses to pathogens and have raised concerns that a familiar quantitative assay is wrong. The most exciting of the new advances is the MHC tetramer. Ever since T cells were first identified, there have been attempts to show that they can be individually identified by the specificity of their antigen binding properties. The early quest failed because the ligand was not known to be an MHC-peptide complex. When the T cell receptor was eventually identified, it was ironic that it had still not been shown to bind anything. However, studies with purified receptors and complexes of antigenic peptide and MHC showed that they did bind specifically, but with low affinity and fast off-rates (1). It is now argued that during T cell recognition, the low affinity and fast off-rate are necessary to enable serial contact of each TCR molecule with multiple MHC-peptide ligands (2). Such characteristics were assumed to be too unfavorable for direct staining of T cells.

Using purified I-E^k molecules bound to a pigeon cytochrome c peptide, Davis and Altman prepared a tetrameric complex (3). They used the enzyme BirA, which specifically biotinylates a lysine residue within a 13-amino acid recognition sequence. The BirA recognition tag was engineered onto the COOH terminus of the extracellular domain of one chain of the MHC molecule, allowing it to be enzymatically labeled with biotin and bound to streptavidin. As the latter has four biotin binding sites, this produced tetramers of MHC molecules that bound to cells displaying the appropriate TCR. These multimeric MHC ligands had a far greater avidity for the T cells than the sum of the individual monomeric affinities. This binding can be easily detected by FACS[®] (Becton Dickinson, Mountain View, CA) analysis if a fluorochrome-labeled streptavidin reagent is used. In collaboration with Bell et al. (4) who had folded HLA B molecules for crystallization, the method was then applied to HLA class I molecules (5). Modeling of HLA B8 with streptavidin (Fig. 1) from known coordinates reveals a flexible 12-amino acid tail at the COOH terminus of the heavy chain separating the $\alpha 3$ domain from the biotin by up to 30 Å. Phycoerythrin, the most commonly used fluorochrome, is quite large and is likely to interfere with access of one HLA peptide with the TCR (Fig. 1). Therefore, much of the binding is likely to be trimeric, although tetrameric binding could be possible when the smaller molecule fluorescein is used to label complexes.

Tetrameric complexes of HLA A2 were made with epitope peptides from HIV *gag* and *pol*, and these were shown to bind to appropriate T cell clones and T cells from peripheral blood of HIV infected patients (5). The numbers of blood T cells stained, up to 2% of CD8⁺ T cells, seemed high compared with precursor estimates made using limiting dilution assays (LDAs) in similar patients of 1/4,000–1/20,000 (6). However, the higher figures were in line with estimates made by analysis of direct cytotoxicity assays (7) and by TCR transcript quantitation for immunodominant CTL clones (8, 9). The message, now amply confirmed, was that persisting virus infection maintained a very strong CTL response. For persistent HIV infection, the range is commonly 0.1–2% of CD8⁺ T cells. In persistent simian immunodeficiency virus infection in macaques, Kuroda et al. (10) show that epitope-specific T cells can account for as many 10% of CD8⁺ T cells in the blood. These numbers are reminiscent of the expanded T cell clones bearing particular V β chains reported by this group (11). Even larger expansions of CD8⁺ T cells carrying oligoclonal T cell receptors have been reported by Pantaleo et al. in acute HIV infection (12). In acute infections, these T cell expansions are relatively short lived and decline to lower levels after the virus load falls to a steady state. Ogg et al. have shown in persistently HIV-infected persons that the level of tetramer staining of HIV-specific CD8⁺ T cells correlates inversely with RNA virus load (13). The circulating activated effector T cells could be controlling virus-infected cells by direct lysis and/or release of cytokines and chemokines.

Recently, three papers have examined the CTL response to acute lymphocytic choriomeningitis virus (LCMV) infection. Butz and Bevan (14) measured the numbers of interferon-producing CD8 T cells after stimulation with epitope peptides, treatment with brefeldin A, permeabilization, and intracellular staining with anti-IFN- γ . They showed that nearly all of the CD8⁺ T cell expansion in the spleens of acutely infected mice resulted from growth of antigen-specific T cells; over a quarter of all CD8⁺ spleen cells at the peak response responded to the three immunodominant LCMV peptides in C57/B6 mice. These data were backed up by experiments where known numbers of TCR transgenic T cells were transferred into mice and then expanded by infection of the host. Murali-Krishna et al. (15) found similar expansions using H-2 class I tetramers constructed with immunodominant epitope peptides. Over 50% of splenic CD8⁺ cells were antigen specific in acutely

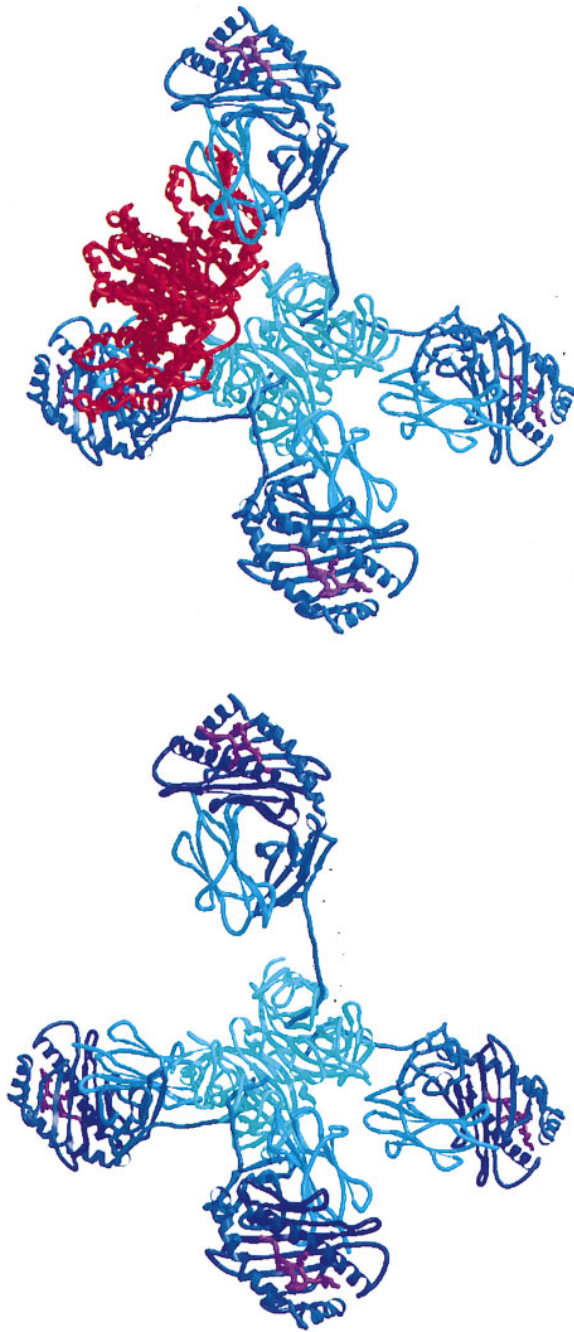


Figure 1. Model of HLA B8-peptide tetramer. *Top*, the complex without the fluorochrome, *bottom*, phycoerythrin bound to the streptavidin. *Light blue*, streptavidin; *dark blue*, B8 heavy chain; *intermediate blue*, beta-2 microglobulin; *purple*, the peptide; *red*, phycoerythrin. The phycoerythrin is represented by the crystallographically determined structure, but in its biologically active form can exist as an even larger trimer of this structure. The potential extended length between the biotin molecule and the $\alpha 3$ domain is 30Å.

LCMV-infected BALB/c and C57BL/6 mice; these T cells were specific for one epitope in the former mouse strain and split unevenly between four epitopes in the latter. When these cells were sorted, almost all made IFN- γ in ELISpot assay, and killing of target cells was greatly en-

riched. The response peaked at day 8, but high levels (up to 10%) of tetramer staining cells were still detectable after a year; the strength of these responses followed the known hierarchy of immunodominance. Gallimore et al. (16) also found large-sized expansions of antigen-specific T cells in acute LCMV infection. Using different doses of a rapidly replicating strain of LCMV, it was shown that the size of the antigen-specific CD8⁺ T cell expansion was determined by the antigen load alone. In all of these experiments, the acute LCMV-specific response is so large that there is no need to invoke bystander effects (17); Butz and Bevan (14) showed that adoptively transferred CD8⁺ TCR transgenic T cells for an ovalbumin epitope did not expand on LCMV infection.

In acute infectious mononucleosis in humans, caused by infection with EBV, Callan et al. (18) found that 20 of 21 patients showed expansions of T cells with restricted TCR expression, detected by monoclonal antibodies specific for TCR V β segments. These numbers returned to normal in the months after recovery. Now Callan et al. (19) have shown, using HLA A2 and B8 tetramers, that between 7 and 44% of blood CD8⁺ T cells are EBV specific in this acute infection. As found in acute LCMV infection, the total number of CD8⁺ T cells is greatly increased, almost entirely by antigen-specific cells. The numbers decline slowly, reaching a baseline only after >6 mo. Interestingly, these responses were directed against EBV lytic cycle epitopes, rather than the more familiar latent epitopes expressed on B lymphoblastoid cell lines. The latter CTL responses were much smaller. In chronically infected persons, up to 2% of circulating CD8⁺ T cells are directed against lytic epitopes, implying continuing turnover of the virus in infected epithelial cells. These numbers are similar to the number of HIV-specific CD8⁺ T cells found in many persons infected with HIV (5).

In acute *Listeria* infection in mice, Busch et al. (20) have followed the CTL response to four epitopes in primary and secondary infection. The four responses to *Listeria* showed similar kinetics in the primary and in the secondary infections. One unique observation in this study was that CD8⁺ T cells continued to expand for a short time after the infecting microorganism had disappeared. In this study and that of Butz and Bevan (14), the issue of immunodominance was addressed. The previously defined hierarchy of immunodominant epitopes in memory CTL responses was matched by the number of responding cells in the acute infection. Thus, it appears that immunodominance is set in the acute infection, provided the acutely activated T cells do not burn out. Gallimore et al. (16) showed that with very high doses of infection with LCMV DOCILE, the previously described clonal exhaustion was accompanied by loss of tetramer-binding T cells. Why certain responses dominate in the acute phase is not clear; it may reflect the quantity of peptide on the infected cell surface, but the number of naive CTLs before infection may also be highly relevant. The binding affinity of the TCR to the peptide-MHC complex may also contribute in a major way to the clonal burst size.

All of these studies show that the acute CD8⁺ T cell response is far greater than generally realized previously, although these results were predicted by the findings of expansions of T cells with restricted TCR usage in acute infections with influenza virus, HIV, simian immunodeficiency virus, and EBV (11, 12, 19, 21). Large proliferations of CD8⁺ T cells in acute infections had also been noted previously, but because of the LDA results it was thought that there must be a large bystander effect (22). In these recent studies with tetramers and antigen-stimulated cytokine staining, the expansions can be wholly accounted for by antigen-specific T cells; the possible role of bystander activation in maintaining T cell memory may have to be rethought.

The size of the CD8⁺ T cell response in acute virus infections is impressive. It is possible that the examples chosen represent one end of a spectrum. In the infections so far studied, the infection is widespread and not localized. In contrast, the immune response to lower respiratory tract influenza infection in mice is maximal in the paratracheal lymph nodes (21) and the size of the splenic T cell response may not be so great. The effect of virus dose shown by Gallimore et al. (16) may also mean that the response is more moderate in less severe infections. For example, many acute EBV infections are subclinical and it seems unlikely that these too will evoke such massive CTL responses.

The growth of antigen-specific T cells *in vivo* is impressive. In the murine LCMV infections, the peak rate of division was calculated to be 2–3/day, similar to the rapid growth of B cells in germinal centers (14, 15). In humans, the exact timing of any natural primary infection is hard to determine but the total number of antigen-specific T cells at the peak response is enormous; if the T cells are distributed evenly throughout the body, 40% of peripheral blood CD8⁺ cells means 4×10^{10} in total. Even a more conservative estimate of 4×10^9 cells would require 32 divisions, if derived from a single clone as some of the TCR V β expansion data imply (12, 18). Most responses are probably oligoclonal, but would still need >25 divisions. Given the difficulty in eliciting primary CTL responses *in vitro*, this is remarkable. We still have much to learn about reproducing *in vivo* responses *in vitro*.

The MHC class I tetramer technique appears to be exquisitely antigen specific and highly sensitive. Staining of T cell clones shows no cross-reactions so far. The method easily stains large numbers and can also detect rare cells; FACS[®] analysis of PBMCs is sensitive down to 0.02%. Dunbar et al. (23) found that sorted rare cells showed the correct antigen specificity in an IFN- γ ELISpot assay (24). The sorted cells could be grown as clones and they showed the expected lytic activity. Remarkably few false positive and negative results have been found so far.

Where does MHC tetramer technology leave other techniques of detecting antigen-specific CD8⁺ T cells? The intracellular cytokine staining of epitope-stimulated T cells gives similar results (15). The complication of making the tetramer is obviated, but the method kills the cells. It is

possible to combine the two techniques, double staining cells with tetramer and anti-IFN- γ , after peptide stimulation, brefeldin treatment, and permeabilization (16). The IFN- γ ELISpot assay for peptide-stimulated cells gives similar results and is efficient at detecting low numbers, for instance, influenza virus-specific responses, long after the virus has departed (24).

Real questions are posed about the value of the LDA. The numbers of precursors detected by LDA are often 50–500 times lower than those detected by the other methods (10). Does this mean the LDA is worthless? One possible explanation is that only a minority of the cloned T cells can actually kill; however, functional assays on sorted tetramer-binding cells argue against this. Another major difference between the LDA and the direct detection assays, such as tetramer staining, is that the LDA depends on cell division. In the informative range of the assay, each microtiter tray well should contain one clone. To detect a cytolytic response, measurable killing is needed, often 10% specific lysis. CTL clone data indicate that this would need an effector/target ratio of $\sim 0.5:1$; using 4×10^3 target cells, 2×10^3 CTLs would be needed in the wells to score positive, i.e., 11 divisions from a single precursor. Bulk culture assays, which are semiquantitative, need a similar number of divisions. Therefore, the low number of T cells detected in acute virus infection indicates that only a minority of antigen-specific T cells, <1%, have made this number of divisions *in vitro*. In acute infections, most of the expanded CTLs are destined to die by apoptosis; this may also be true of the T cells activated at a lower level by persisting virus. Only when the virus has been gone for some time (years in humans) do the LDA and tetramer assays come close (23). The LDA may therefore give a meaningful figure of T cells with long-term growth potential. Subtle differences in the strength of signal given by the TCR and accessory molecules could determine the rate of growth such that only those receiving the strongest signal show up in the LDA. However the assay is beset by technical difficulties and assumptions about the cutoff. The LDA needs careful reassessment in the light of these recent findings.

The differences between the numbers obtained by the different assays could be reconciled as shown in Fig. 2. There is a problem of terminology: Should T cells be described by their function or antigen binding properties? Although there is agreement between tetramer staining and staining for IFN- γ , it is harder to show that all the detected cells can kill. Nevertheless, the term effector CTL (eCTL) is used to describe expanded CD8⁺ antigen-binding T cells that have limited growth potential (*in vitro*, at least); they are assumed to have some effector function such as lysis, cytokine release, or both, but it is recognized that some T cells in this category could be exhausted. Gallimore et al. (16) showed that in some situations where there is excessive antigen, antigen-specific T cells can be detectable using tetramers, although these cells exhibited a diminished capacity to produce IFN- γ and lytic activity *in vitro*. Thus, quantitation of antigen-specific cells may not always accurately reflect the antiviral potential of the expanded population.

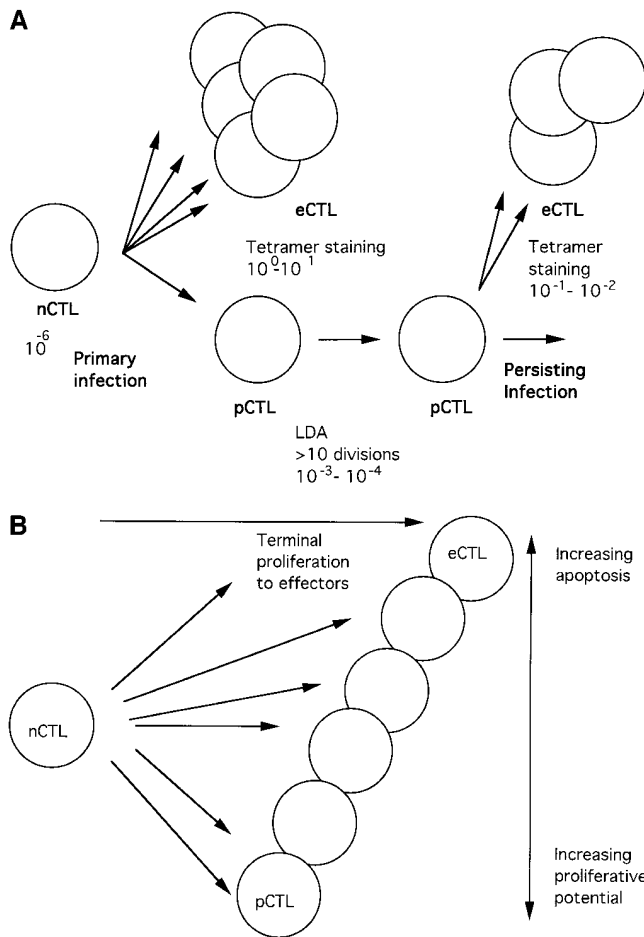


Figure 2. (A) Scheme of the life history of CTL clones specific for a single epitope. nCTLs are rapidly triggered by primary virus infection to generate very large numbers of effector eCTLs that are functional but doomed to die by apoptosis. A small subpopulation takes an alternative route and maintains their growth potential; these pCTLs are detectable by LDA and carry long-term memory. (B) Alternative scheme for the life history of CTLs. On contact with antigen, CTLs proliferate at different rates. At the extremes, greatly expanded eCTLs apoptose and those that proliferated very little persist as long-term memory cells. Many cells fall in between. This model predicts that the clonal composition of eCTLs and pCTLs would be different. A theoretical problem with this model is that cells that divide poorly in the primary response have the greatest growth potential and have to give rise to rapidly dividing daughter cells on reinfection.

Effector CTLs are fas-positive and likely to die by apoptosis. The term precursor CTL (pCTL) is used in Fig. 2 A to describe only those T cells, detected by LDA, with growth potential. It is possible that there is a continuum of expanded cells having a growth potential of between 0 and 25 or more divisions; if so, the eCTL and pCTL categories would refer only to the extremes (Fig. 2 B). However, this model would predict that the acute immune response and long-term memory response might have quite different clonal composition and even different specificities. The data collected so far suggest the opposite (10, 14, 15, 20, 25).

CTL clones that have not yet made contact with antigen would be described as naive CTLs (nCTLs). Both eCTLs and pCTLs belong to the memory pool. However, it could be argued that only those with true long-term growth potential are the true memory cells, the cells that can survive long after antigen has gone. The differences between nCTLs and pCTLs include their absolute numbers and expression of certain surface glycoproteins such as CD45 isomers. The growth potential of some naive T cells is very impressive. It is curious that it is so difficult to reproduce this in vitro, but with possible exceptions (26). Primary CTL responses in vitro have not been easily obtained and it may take several weeks to culture rather poorly growing clones. The findings of these recent papers should spur efforts to stimulate rapid massive CTL responses in vitro. Attention needs to be paid to the epitope chosen as well as the antigen-presenting cells, probably dendritic cells, the local environment, and the cytokine milieu required.

Finally, Are these very large primary CD8⁺ T cell responses necessary or is there an overreaction? For persistent HIV infection, Ogg et al. have shown an inverse correlation between the level of tetramer positive CD8 T cells and virus load (13). In the systemic infections studied, the number of virus-infected cells must be very high. The ratio of reactive T cells to infected target cell would be expected to favor lysis of infected cells before they release new virus particles (27). A degree of overresponse, provided it is controlled by apoptosis, may well be advantageous; it is reassuring that CD8⁺ T cell malignancies are rare. These common massive T cell expansions must be well managed.

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