MHC Tetramer Analyses of CD8+ T Cell Responses to HIV and SIV

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ABSTRACT

MHC tetramers are reagents used for direct *ex vivo* analysis of the frequency and phenotypes of antigen-specific T cells by flow cytometry. The tetramer staining assay is purely physical—relying only upon the interaction between the tetramer reagent and T cell receptor clusters (and possibly co-receptors) on the surface of T cells—and reduces to an absolute minimum the *in vitro* manipulation of the sample before detection of the antigen-specific population. The assay does not require *a priori* assumptions of the class of functional responses (*e.g.* cytokine profiles), and is therefore likely to provide the most complete method for detection of the magnitude of an antigen-specific response, while other methods can be applied to examine functional subsets within the total antigen-specific population. The method is extraordinarily robust, and can detect antigen-specific populations at frequencies as low as 1:5,000 CD8+ T cells (or approximately 1:50,000 PBMC). All of these factors combined suggest that MHC tetramer staining will play an essential role in evaluation of T cell immune responses in future HIV vaccine trials and in patients infected with HIV.

INTRODUCTION

Until recently, most quantitative measurements of CD8+ T cell immune responses were made with either bulk *in vitro* stimulation with antigen followed by a chromium release assay to measure cell killing mediated by cytotoxic T lymphocytes (CTL), or by adaptation of this basic procedure to determine the frequency of CTL precursors (CTLp), known as limiting dilution analysis (LDA). Because the chromium release assay is relatively insensitive and cannot detect many fewer than 2000 activated antigen specific CTL (McMichael & O'Callaghan, 1998), both assays require *in vitro* stimulation of cells taken directly *ex vivo* to expand the antigen-specific population and to induce these cells to differentiate into CTL effector cells. The specific responses detected in bulk CTL assays provide at best a semi-quantitative description of the immune response as it actually exists *in vivo*, while LDA is now known to underestimate the true frequency of antigen-specific T cells by 10–500 fold. This underestimate is a result of the very stringent operational definition of a CTLp in the LDA; to be detected in the assay, a single cell must be induced to go through at least 11 rounds of cell division and the progeny must develop cytolytic functions (McMichael & O'Callaghan, 1998).

The bulk CTL assay and LDA may therefore be fairly said to reflect *in vitro* generated activities, and extrapolation of these results back to the state of the immune system *in vivo* is likely to be imprecise at best. There was therefore a strong need for assays—preferably simple and rapid—which could detect antigen-specific T cells at the single-cell level, directly *ex vivo*, without the need for *in vitro* expansion. The MHC tetramer assay (Altman *et al.*, 1996) (and a related method using MHC-Ig chimeras (Greten *et al.*, 1998; Hamad *et al.*, 1998)) was developed in response to this need. The method is complemented by two recently developed functional assays—antigen-specific intracellular cytokine staining (Murali-Krishna *et al.*, 1998) and the cytokine ELISpot assay (Lalvani *et al.*, 1997) —that require *in vitro* stimulation *but not expansion* of an antigen-specific population prior to measurement.

Early attempts to identify antigen-specific T cells by flow cytometry focused on systems where the repertoire of the T cell response was highly restricted. These T cells could by identified by staining with antibodies against specific Vα and Vβ T cell receptor (TCR) domains found in the responding population, in conjunction with markers associated with antigen-experienced cells such as CD62L and CD44 (McHeyzer-Williams & Davis, 1995). We, and others, (Doherty *et al*., 1996), recognized that T cell responses using highly restricted Vα/Vβ repertoires were likely to be exceptional, and that a more general approach to the problem was called for. Instead of using anti-V region antibodies as a surrogate marker for antigen-specificity, we (Michael McHeyzer-Williams, Mark Davis, JDA) decided to try to stain T cells directly with the antigen that they recognize, namely MHC/peptide complexes (McHeyzer-Williams et al., 1996). These efforts failed, and it soon became apparent why: in order to

maintain self-tolerance, the affinity of MHC/peptide for the TCR was too low to use as an immunological staining reagent (Matsui *et al*., 1991; Matsui *et al*., 1994). We then sought methods to add an avidity component to the reagents, thereby decreasing the effective dissociation rate of the MHC/peptide based stain.

To produce an MHC/peptide based stain with avidity for T cells, we turned to streptavidin/biotin chemistry. Streptavidin (and the related avidin molecule) is a protein composed of four identical subunits, each with an exceedingly strong affinity for biotin. We reasoned that if we could biotinylate an MHC/peptide complex and mix that preparation with streptavidin at a 4:1 molar ratio, we would produce a tetrameric complex that might have strong avidity for antigen-specific T cells. Our first attempts to use MHC/peptide complexes chemically biotinylated by treatment with an N-hydroxy-succinamide (NHS) derivative of biotin failed because the chemical modification was essentially random and the biotin-MHC/peptide complexes therefore bound to streptavidin in random orientations, some of which apparently could not interact with the TCR on the surface of cells.

The key to producing a reagent that effectively stained specific T cells was to use a method for attaching the biotin that was much more specific than chemical biotinylation with NHS-biotin and that targeted the biotin residue to a site at the carboxy-terminal end of the soluble domain of the MHC, as far away as possible from the TCR interaction site. To accomplish this, we prepared MHC molecules that were fused to a 15 amino acid peptide that is a substrate for biotinylation by an enzyme found in *E. coli* known as BirA (Schatz, 1993); we refer to this peptide as a BSP, for BirA substrate peptide (Altman et al., 1996). Following methods developed by Garboczi *et al*., (Garboczi *et al*., 1992) we express the class I MHC heavy chain-BSP fusion and β2 microglobulin subunits as insoluble, misfolded inclusion bodies in *E. coli*, and then mix them together under folding conditions *in vitro* in the presence of a specific peptide ligand to produce MHC/peptide complexes. These molecules are purified by size exclusion chromatography, enzymatically biotinylated using biotin, MgATP and the MHC-BSP/peptide complexes as substrates for the BirA enzyme (available from Avidity, Denver CO), and purified again on high resolution anion exchange columns (Altman *et al*., 1996). The biotinylated MHC/peptide complexes are then mixed with fluorescently labeled streptavidin at a 4:1 molar ratio to produce MHC tetramers, as shown in Figure 1.

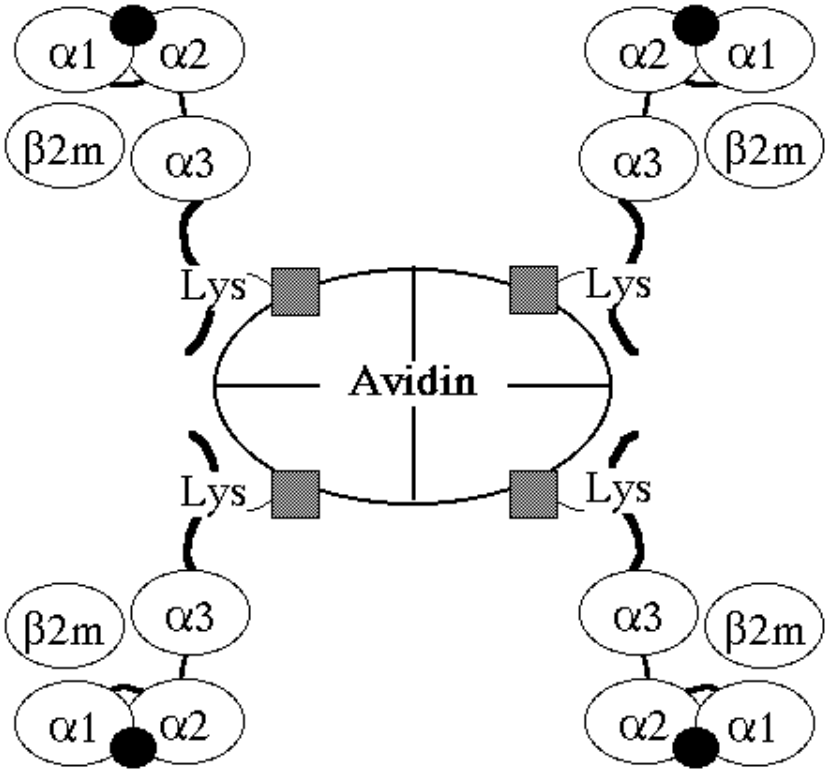

Figure 1. Cartoon of a class I MHC/peptide tetramer. The domains of the MHC subunit are labeled in the figure, the peptide epitope is shown in filled black ovals, biotin residues are shown in gray squares, the BirA substrate peptide is shown as a tail connected to the a3 domain of the MHC, and the lysine residue in the BSP that is biotinylated is explicitly labeled.

MHC tetramers provide a tool for measurement of a scalar quantity, the frequency of an antigen-specific T cell population. We were initially concerned that tetramer staining might fail to detect a significant fraction of T cells during both acute and chronic immune responses due to down regulation of the TCR in response to stimulation of the T cell with antigen *in vivo* (Valitutti *et al*., 1995), or that MHC tetramers might fail to stain T cells with low—but still functionally relevant—avidity for antigen. Two arguments can be advanced to allay these

fears. First, there are no examples where the frequency of antigen-specific T cells determined by functional assays exceeds the frequency determined by tetramer staining, suggesting that MHC tetramers give a complete accounting of the T cell immune responses for the appropriate MHC/peptide combination. Second, the sheer magnitude of the acute immune response that is detectable by tetramer staining suggests that the fraction of specific cells not detected with the tetramer must be very low. Two examples suffice. During the acute immune response to lymphocytic choriomeningitis virus (LCMV) in BALB/c mice, 55% of all CD8+ splenocytes are stained with the L^d /NP118 tetramer (Murali-Krishna *et al.*, 1998), while during the acute primary human immune response to Epstein Barr Virus, up to 44% of all circulating CD8+ PBL were stained with an HLA-B8 tetramer containing a peptide from the EBV lytic phase protein BZLF1, HLA-B8/RAK (Callan *et al.*, 1998), and almost all of these express high levels of CD38, suggesting that they are highly activated.

Distinct subsets are found within the population defined by tetramer staining, which might be described by a *phenotypic matrix*. The dimensionality of this matrix is indeterminate and can be quite large. For example, one dimension can be assigned for the expression level of each cell surface marker of interest, such as CD62L, CD45RA, CD38, etc. Separate dimensions might also be assigned for functional activities within the total population. Examples might include subsets which score as CTLp in LDA or cells which can be induced to secrete cytokines after short term *in vitro* stimulation. Ironically, it is difficult to assess the distribution of the most familiar functional activity attributed to CD8+ T cells, namely their cytolytic function, as CTL activity is commonly measured for a population of cells and few investigators even attempt to measure CTL activity at the single cell level.

In order to obtain a more precise quantitative measurement of T cell immune responses with MHC tetramers, a price has to be paid. In contrast to measurements with bulk CTL assays or LDA, detection of T cell immune responses with tetramers requires precise knowledge of the optimal T cell epitope and its restriction element. When working with outbred populations (*e.g.* rhesus macaques or human subjects), it is therefore necessary to obtain medium-to-high resolution MHC typing information before suitable reagents can be selected or newly prepared; it is not yet clear if tetramers prepared with one MHC subtype (*e.g.* HLA-A*0201) will cross-react with T cells generated in responses to individuals with closely related subtypes (*e.g.* other HLA-A2 subtypes). In addition, in vitro folding of the MHC/peptide complexes is not tolerant of extensions or truncations of the optimal peptide ligand. The tetramer assay is therefore not suited to the early stages of investigation of the breadth of a T cell immune response. And although it is theoretically possible to use MHC tetramers to test motif-based predictions of CTL epitopes, the ELISpot or intracellular cytokine staining assays are much better suited to the task of screening large numbers of candidate epitopes for recognition by T cells. Finally, expression plasmids suitable for the preparation of MHC tetramers are available for only a limited number of human and rhesus macaque class I MHC alleles (constructs for all classical class I alleles in mice of the H-2b, d, and k haplotypes are available), although the constructs are easy to prepare this is not expected to be a serious limitation in the near future.

Assuming that acquisition of reagents is not a factor, the tetramer assay is the most rapid and direct method for determining the magnitude of a T cell response. In contrast to the ELISpot assay, the antigen-specific cells are easily counted by a computer (although semi-automated image processing of ELISpot plates may soon be available). Unlike the ELISpot assay, the tetramer method does not require accurate counts of the number of input cells, or separate measurements to determine the fraction of the total population that is represented by CD8+ T cells. These advantages also apply to the intracellular cytokine staining, but that method is significantly more labor intensive than the tetramer staining method, which may be a decisive disadvantage in evaluation of large scale vaccine trials. On the other hand, both cytokine assays do provide important functional data that is not available when only the tetramer assay is employed.

Most—but not all (McHeyzer-Williams *et al.*, 1996; Crawford *et al.*, 1998; Gutgemann *et al.*, 1998; Hamad *et al.*, 1998)—of the work with multimeric MHC molecules has focused on CD8+ T cells which recognize peptides bound to class I MHC molecules. This is because simple and efficient protocols have been developed for producing class I MHC/peptide complexes from subunits produced in E. coli, and the basic protocol can be applied to nearly every classical class I MHC allele without modification. In contrast, although several expression systems have been described for soluble class II MHC (Wettstein *et al.*, 1991; Stern & Wiley, 1992; Altman *et al.*, 1993; Kozono *et al.*, 1994; Kalandadze *et al.*, 1998; Hamad *et al.*, 1998; Scott *et al.*, 1998), expression of soluble class II MHC appears to vary from allele to allele, and to increase the stability of the molecules, most investigators expressing class II MHC are now following the lead of Kappler and colleagues (Kozono *et al.*, 1994) and are preparing expression constructs with the peptide ligand covalently attached to the β chain of the class II molecule, limiting the general applicability of any

individual expression construct. In the remainder of this review, we will focus on features of MHC tetramer analysis and results that are most relevant to the study of immune responses to HIV, and to the development of an HIV vaccine.

MAGNITUDE OF T CELL IMMUNE RESPONSES

With rare exception (Borrow *et al.,* 1997), measured frequencies of HIV-specific CTLp do not exceed 1:1000, yet data existed suggesting that the true frequency of HIV-specific CD8+ T cells was in many cases significantly greater than this number. First, there is the frequent observation of direct *ex vivo* CTL activity in PBMC from a high percentage of HIV-infected patients suggesting HIV-specific CTL at frequencies approaching 1:100 per CD8+ T cell (Gotch *et al.,* 1990). This was confirmed by estimation of the frequency of an HIV-specific T cell clone in PBMC using molecular methods to estimate the frequency of TCR transcripts containing a CDR3 sequence from a well characterized CTL clone (Moss *et al.,* 1995). It was these data which first led us to apply the tetramer technology to immune responses against HIV (Altman *et al.,* 1996). Tetramer staining has confirmed that in chronically infected, drug naïve patients, the frequency of HIV-specific CD8+ T cells can be commonly found in the 1–4% range (Ogg *et al.,* 1998; Wilson *et al.,* 1998; Ogg *et al.,* 1999).

Ogg *et al.,* have reported that there is a positive correlation between the frequency of tetramer staining cells and direct *ex vivo* lytic activity (Ogg *et al.,* 1998), suggesting that the tetramers provide an accurate count of the frequency of circulating effector CTL (CTLe). However, our data from studies of CD8+ T cell responses in mice suggests that this result should be interpreted with caution. First, we and others have found that it is in general not possible to detect IFNγ production by antigen-specific T cells—even when they are sampled at the peak of an acute immune response—without stimulating the cells *in vitro* with peptide (Flynn *et al.,* 1998; Murali-Krishna *et al.,*1998) or PMA/ionomycin (Gallimore *et al.,* 1998) This result might itself be an *in vitro* artifact, but at a very minimum, it does suggest that at least one effector phenotype—cytokine secretion—may be rapidly reversed without cell death. Second, we have found that infection of LCMV-immune mice with wild type vaccinia did not alter the total number of LCMV-specific CD8+ splenocytes—and in fact decreased their frequency due to expansion of vaccinia-specific cells—but did increase the direct *ex vivo* CTL activity against targets presenting LCMV antigens. This suggests that there is a significant population of LCMV-specific cells that are not effectors before infection with the heterologous vaccinia, and that are then "activated" in a bystander fashion to become effectors. Finally, we have recently found that chronic infection of CD4-deficient mice with LCMV can lead to induction of unresponsiveness in a sizable population of LCMV-specific CD8+ T cells identified by tetramer staining (Zajac *et al.,* 1998), a result that has significant implications for the character of the CD8+ T cell response during chronic infection with HIV (Kalams & Walker, 1998). Development of an assay to detect cytolytic function at the single-cell level would greatly contribute to a resolution of this controversy.

A more important result from the paper by Ogg *et al.,* was the observation of a highly significant inverse correlation between the frequency of HLA-A2/gag or A2/gag + A2/pol tetramer positive cells in fresh peripheral blood and the viral load (Ogg *et al.,* 1998). These data strongly suggest that HIV-specific CD8+ T cells play an important role in the control of HIV infection, although it remains difficult to determine which effector functions of the specific CD8+ population are most responsible. Given the importance of this result, it is essential that it be confirmed in patients making immune responses that are restricted by HLA alleles other than HLA-A*0201. One factor which may complicate this experiment is the possibility that the responses that are detected by tetramer staining may not always be the dominant ones in every individual sharing that HLA allele. This points to one of the limitations of the tetramer technique—tetramer staining provides precise measurements of a very specific response, while revealing nothing about responses that are specific for additional epitopes in the same virus.

Tetramer analyses of acute immune responses to HIV in infected humans have not been published, but existing data suggest that the acute immune response against HIV may be similar in magnitude to the acute response seen against other systemic infections such as LCMV in the mouse (Murali-Krishna *et al.,*1998) and EBV in humans (Callan *et al.,*1998). First, there are often large expansions of CD8+ T cells with a restricted Vβ repertoire seen during the acute phase of HIV infection (Pantaleo *et al.,*1994), similar to the restricted Vβ expansions seen following acute EBV infection (Callan *et al.,*1996). Second, Borrow and colleagues used limiting dilution analysis to measure a monospecific acute immune response against HIV at a frequency of 1:15 PBMC.

Obtaining a detailed analysis of the kinetics of the acute HIV-specific CD8+ T cell response in humans is difficult because of the uncertainty in determining the time of infection. Analysis of the CD8+ T cell immune response following infection of rhesus macaques with SIV or chimeric SHIV will undoubtedly be a useful model for studying acute responses to immunodeficiency

viruses (Reimann *et al.*,1994). MHC tetramer technology has been used to measure the magnitude of the CD8+ T cell response to the Mamu-A*01-restricted response to the SIVgag-p11C(C-M) epitope (Evans *et al.*,1997) in A*01+ macaques chronically infected with SIVmac 251 (Kuroda *et al.*,1998). In three chronically infected animals, the frequency of cells staining with the Mamu-A*01/p11C(C-M) tetramer ranged from 0.9–10.3% of fresh CD3+CD8$\alpha\beta$+ T cells in PBMC, even while the frequency of CTLp with the same specificity measured by LDA never exceeded 1:4000 PBMC (Kuroda *et al.*,1998). Even more exciting were the results in animals vaccinated with a recombinant modified vaccinia Ankara (MVA) expressing the SIV gag and pol genes (Seth et al, 1998). All Mamu-A*01+ animals (n=4) had Mamu-A*01/p11C(C-M) tetramer binding T cells 10 days after a second vaccination with the recombinant MVA, with frequencies ranging from 0.6–4.9% of all CD8 antibody positive T cells in PBMC (Seth *et al.*,1998). This result suggests that MHC tetramers will be an extremely valuable tool for measuring vaccine induced responses, even when the recombinant vaccine is a replication-defective virus (Carroll & Moss, 1997; Seth *et al.*, 1998).

The kinetics of the CD8+ T cell immune response in rhesus macaques to SIV is expected to vary with the strain of virus used and with the route of infection. We have begun to use Mamu-A*01/p11C(C-M) tetramers to measure immune responses in animals infected intravenously with SIVmac Δ3 (Desrosiers 1992), SHIV89.6 (Reimann *et al.*, 1996b), SHIV89.6P (Reimann *et al.*, 1996a), as well as animals infected intrarectally with SIVmac 239 or SIVmac 251 (Altman *et al.*, unpublished). Preliminary results suggest that, as expected (Reimann *et al.*, 1994), the peak of the acute immune response following intravenous infection occurs 2–3 weeks post infection, and that responses against the most attenuated strains (*e.g.* SIVmac Δ3) tend to be the weakest, with Mamu-A*01/p11C(C-M) tetramer binding cells peaking at levels between 0.6–1% of CD3+CD8+ T cells.

Extensions of these initial studies in the rhesus macaque model are currently limited by the small number of well characterized HIV or SIV epitopes and their restricting MHC alleles in rhesus macaques (Charini & Letvin, 1997). The MHC-related problems are being addressed by the development powerful molecular typing methods (D. Watkins, personal communication) and the establishment of breeding colonies of MHC-defined animals. At the same time, more epitopes will be characterized using MHC-peptide binding motif based approaches (Allen *et al.*, 1998a) and classical eptiope mapping approaches starting with recombinant vaccinia viruses, as well as the application of new methods for detection of CD8+ T cell responses in fresh PBMC samples by stimulation of cytokine production by libraries of overlapping peptides (Kern *et al.*, 1998).

LONGITUDINAL ANALYSES

An extensive natural history study of the frequency and phenotype of MHC tetramer-positive T cells following infection with HIV or SIV has not yet appeared. Given the current standard of care, such studies will almost certainly be done retrospectively, using frozen samples from an established cohort such as the Multicenter AIDS Cohort Study (MACS). Several labs have shown that tetramer staining works well on frozen cells (Ogg *et al.*, 1998), suggesting that the results of such studies may be regarded with confidence.

The most interesting longitudinal analyses that have appeared so far have examined the frequencies and phenotypes of HIV-specific, tetramer-positive cells following viral load reduction with highly active antiretroviral therapy (HAART) (Ogg *et al.*, 1998; Ogg *et al.*, 1999), complementing previous work examining virological and immunological responses to perturbation of an established steady state (Ho *et al.*, 1995; Wei *et al.*, 1995). Viral loads for all patients in this cohort were suppressed below the limits of detection throughout the course of the study. Immediately following initiation of HAART, the frequency of tetramer binding CD8+ cells fluctuates rapidly, probably due to redistribution of antigen-specific CD8+ T cells from secondary lymphoid organs to the peripheral blood (Ogg *et al.*, 1999), reminiscent of previously observed fluctuations in total CD8+ T cells observed following initiation of HAART in some patients (Autran *et al.*, 1997). After this initial phase, there is a lengthy phase in which the frequency of tetramer-binding cells decays exponentially, with a median half life of 45 days (Ogg *et al.*, 1999). In one patient, the frequency of tetramer positive cells declined from 2.75% of CD8+ T cells at the start of therapy to 0.47% 18 months later. The decay in the frequency of tetramer binding cells is much more gradual than the decay seen after resolution of acute infection with LCMV in a mouse model (Murali-Krishna *et al.*, 1998), where the virus is cleared by 8 days post-infection, and the total number of antigen-specific cells reaches a steady level by day 30 that is maintained for at least one year. The difference may reflect different lifespans of memory T cells in the two species, or it is possible that the decay of HIV-specific cells after suppression of viral loads to undetectable levels is balanced by stimulation of the antigen-specific population by an unidentified reservoir of HIV-infected cells. It will be interesting to one day compare the rate of decay of HIV-specific

CD8+ T cells after initiation of HAART with the decline of an antigen-specific population after resolution of an acute infection by a virus that does not persist.

PHENOTYPIC ANALYSIS

While MHC tetramer analysis is purely a physical assay, analysis of the phenotypes of the tetramer binding cells may suggest something of their function *in vivo* (Altman *et al.*, 1996). A large number of antibodies against cell surface markers have been used to further analyze the phenotype of antigen-specific cells. Almost by definition, if a population of CD8+ T cells is detected by tetramer staining, that population must be "antigen-experienced" because the frequency of antigen-specific cells in naïve subjects is too low to be detected by tetramer staining (Ogg *et al.*, 1998; Seth *et al.*, 1998). Almost invariably, the tetramer positive cells express high levels of the cell adhesion molecule CD11a. In our hands, tetramer positive cells almost always express low levels of the lymph node homing receptor CD62L, although a substantial fraction of EBV-specific CD8+ T cells in some patients with acute infectious mononucleosis (AIM) retained the same high-level expression of CD62L normally associated with naïve T cells (Callan *et al.*, 1998). In the early phase of most immune responses, the tetramer positive cells are CD45RO+ and negative for the reciprocal marker CD45RA, but over time tetramer positive cells with the CD45RO-CD45RA+ phenotype associated with naïve cells do accumulate (Callan *et al.*, 1998) (Altman *et al.*, unpublished). The patterns of expression of CD28, a costimulatory molecule that is a ligand for B7-family molecules found on antigen presenting cells, remain poorly understood. Naïve T cells are thought to express high levels of CD28, and the large number of CD28- CD8+ T cells which accumulated during development of HIV disease have been observed to have shortened telomeres, suggesting that the population has already gone through many rounds of cell division and reached a state of replicative senescence (Effros *et al.*, 1996). Consistent with this observation, Callan *et al.*, have observed that in one individual, the fraction of antigen-specific CD28- cells increases with the increase in frequency of the cells which bind a distinct tetramer (Callan *et al.*, 1998). On the other hand, we have shown that HIV-specific, CD8+ CD28- tetramer+ cells can usually be induced to proliferate *in vitro* (Gray *et al.*, 1999).

The two most interesting phenotyping markers that have been examined are HLA-DR and CD38, because these molecules are thought to be the best surrogate markers for the activation status of a CD8+ T cell; of the two, CD38 expression on tetramer positive cells has been analyzed in more detail (Callan *et al.*, 1998; Ogg *et al.*, 1998; Ogg *et al.*, 1999). CD38 expression on tetramer positive cells appears to correlate with antigen levels *in vivo*. During acute EBV infection, nearly all tetramer positive cells are CD38+, while 3 years after resolution of the acute infection, only 10% of tetramer positive cells remains CD38+. In HIV-infected patients, suppression of viral loads following initiation of HAART not only causes a decline in humans in the total frequency of tetramer positive, HIV-specific CD8+ T cells, but it also causes a reduction in the levels of CD38 expressed on those tetramer positive cells which remain (Ogg *et al.*, 1998; Ogg *et al.*, 1999). When cells from chronically HIV-infected patients are stimulated with peptide antigen for 1–2 weeks *in vitro*, the expanded tetramer population is strongly CD38+, and efficient killing by the cultured cells is observed (Gray *et al.*, 1999).

In addition to using MHC tetramers to examine the phenotype of antigen-specific cells, it has also been possible to costain antigen-specific CD8+ T cells with MHC tetramers and antibodies against TCR Vβ families in both mouse models (Busch *et al.*, 1998; Sourdive *et al.*, 1999) and in HIV-infected humans (Wilson *et al.*, 1998). This was initially surprising, since the tetramer and the Vβ antibody are binding to the same protein domain, and competition for binding sites might have been expected. On the other hand, the tetramer should bind to the most variable portion of the TCR, focusing on the CDR3 regions, while TCR Vβ antibodies should recognize features that are conserved on all T cells expressing that particular Vβ segment. Costaining with MHC tetramers and Vβ antibodies has already proven extremely valuable for analyzing the specificity of T cells in individuals with large Vβ expansions (Callan *et al.*, 1996; Callan *et al.*, 1998; Wilson *et al.*,1998).

PROSPECTS FOR VACCINES

MHC tetramers are ideally suited for the analysis of vaccine induced responses, provided that the CD8+ T cell responses induced by the vaccine are strong enough. The sequences of the HIV antigens delivered by almost any vaccine should be well known, and it is therefore certain that a vaccinated individual—expressing a known HLA allele—will have been exposed to a well characterized epitope; this is in contrast to the analysis of HIV-specific T cell responses in infected patients, some of whom may have been infected with a strain of virus that does not contain the epitope of interest. In addition, the tetramer assay is extremely rapid and therefore might be incorporated into the routine analysis of even large scale phase III clinical trials, providing the HLA typing information is available. As noted above, an actual candidate vaccine—recombinant MVA expressing HIV or SIV antigens—has already been shown

to induce CD8+ T cell responses that are easily detectable with tetramers. If it becomes clear that HIV-specific CD8+ T cells are an important correlate of protection, the responses induced by the MVA vectors may in the future serve as a standard against which other immunogens are measured. It is already clear that these vectors are capable of inducing acute CD8+ T cell immune responses that are equal or greater in magnitude to those induced by strains of live attenuated SIV (Altman *et al.*, unpublished). It remains to be seen if other experimental vaccines, including naked DNA or recombinant fowl pox vectors are also capable of inducing tetramer-detectable immune responses, or how these strategies might best be combined.

SUMMARY AND FUTURE QUESTIONS

MHC tetramer technology has now reached a stage of maturity where it is clear that reagents can be engineered to detect CD8+ T cell immune responses to nearly any antigen of interest, and that these reagents will be specific and have minimal cross-reactivity. Among the questions that will concern readers of this volume are the following. How can we best use the database of defined CTL epitopes as a guide for preparing new reagents to analyze HIV-specific responses in infected patients and in vaccinees? How robust are the responses against epitopes listed in this database? How often can we account for failure to respond to a previously characterized epitope through analysis of the viral sequence, *i.e.* by demonstrating that non-responders were never (or are not now) exposed to that epitope? And how do responses against one epitope affect the generation of responses against a second epitope? How does the particular combination of immune response genes (*i.e.* HLA alleles) in the host affect the response to HIV or any other pathogen? These are complicated questions, but they have important implications for the development of vaccines, and application of the tetramer technology should greatly facilitate the search for answers to these questions.

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