Commentary

Assessing CD8 T Cell Number and Dysfunction in the Presence of Antigen

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CD8 T cells proliferate and differentiate into cytokine-secreting cytolytic effectors on encounter with viral antigens. As virus is cleared the activated T cells undergo apoptosis, but some survive and enter the memory pool where they persist indefinitely, slowly dividing in a relatively quiescent state until reactivation on reexposure to the antigen (1). The dynamics of this whole process can be variable and change as a consequence of the antigen load, the diversity of the available T cell repertoire, and the age of the host. During persistent infections, T cells and antigen coexist in an interactive environment, leading to a continued evolution of T cells that often become dysfunctional. New technologies with avidin-linked MHC tetramers, chimeric IgG–MHC dimers, and peptide-induced intracellular cytokine assays have allowed for the identification of multiclonal populations of antigen-specific T cells and are now showing how T cells can be heterogeneous in their expression of surface antigens and exist in dramatically different functional states. Under certain conditions, antigen-specific T cells may lack effector activity, and under other conditions, fully functional T cells may lack reactivity with MHC tetramers or dimers, leading one to conclude that monitoring T cell responses by a single technique may lead to misconceptions of the nature of an ongoing T cell response.

Functional Antigen-specific T Cells. In mice acutely infected with a moderate dose of lymphocytic choriomeningitis virus (LCMV), the frequencies of CD8 T cells detected by MHC tetramers and MHC dimers correlate well with the total number of activated CD8 T cells and with the frequencies of antigen-specific cells detected by peptide-induced intracellular IFN-γ assays (2–4); these same effector cells are highly cytotoxic against targets pulsed with LCMV-encoded peptides. The frequencies of T cells that seed the memory pool are functions of the clonal burst size of T cells during the acute response (5); this burst size is very high in the LCMV system, and, as a consequence, 10–15% of the spleen CD8 T cells remain specific for LCMV in the memory pool (2, 4). In this memory state, there remains excellent concordance among the MHC tetramer, MHC dimer, and intracellular IFN-γ assays (2, 4). The memory pool specific for each of these LCMV epitopes remains functional and stable in the absence of immune system perturbance, but other infections can disrupt this memory pool by displacing the LCMV-specific T cells with memory T cells of other specificities (4).

Dysfunctional Antigen-specific T Cells. The concordance in systems analyzing T cell number and function is lost under conditions of high doses of disseminating strains of LCMV that "clonally exhaust" T cells and lead to persistent infections (6). The nature of clonal exhaustion has not been well understood, though it had been speculated that the overwhelming amount of antigen may drive the T cells into apoptosis by activation-induced cell death (AICD; reference 1). T cells from mice acutely infected with LCMV become highly susceptible to AICD in vitro, and LCMV-specific transgenic T cells disappear from mice infected under clonally exhausting conditions or after injecting mice with high doses of immunogenic peptides (6–9). However, the use of MHC-tetramers has revealed a more complicated process, as nucleoprotein (NP)396-specific T cells first become dysfunctional and then disappear, perhaps by AICD, whereas glycoprotein (GP)33-specific T cells become anergized but remain present in the host for a long duration (10). In this case, the GP33-specific T cells lack cytotoxic activity or the ability to secrete cytokines. These otherwise anergic cells expressed normal activation markers ($CD62L^{lo}$, $CD44^{hi}$, $IL\text{-}2R^{hi}$) and appeared to be proliferating in vivo, as shown by uptake of bromodeoxyuridine (10). In the absence of CD4 T cells, this anergization occurred much more rapidly, indicating that T cell exhaustion may be in part regulated by CD4 T cells (10). Why T cells of one specificity should disappear whereas those of another specificity remain might relate to the degree of antigen stimulation, as the NP epitope is of higher affinity than the GP33 epitope to the MHC and TCR (11), and substantially more NP than GP is made in LCMV-infected cells, particularly during conditions of persistent infection (12). T cell activation in regards to cytokine production, proliferation, and cytolytic activity all require different thresholds of peptide stimulation (13), so it seems likely that the anergization and apoptotic processes will also be threshold dependent, leading to the possibility of T cells coexisting at different stages of anergy.

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Studies on the acute simian immunodeficiency virus (SIV) infection of macaques have revealed a severe dysfunction of tetramer-staining T cells during the acute infection, under conditions where there is a substantial loss in CD4 T cells (14). Here, CD8 T cells staining with a gag-specific tetramer were cytolytically inactive and failed to produce IFN-γ or IL-2. Some were undergoing apoptosis, as demonstrated by staining with annexin V, whereas the rest were alive but in a dysfunctional state. However, function could be restored after several days of incubation with IL-2, leading the authors to speculate that, like the LCMV system, cytokines produced by CD4 T cells may maintain the function of the CD8 T cells (14).

The findings with LCMV and SIV would suggest that persistent HIV infection, which is associated with a CD4 T cell deficiency, might also be associated with dysfunctional CD8 T cells, and this appears to be the case, though descriptions of the dysfunction have differed. Gray et al., showed that the majority of tetramer-positive HIV-specific T cells in asymptomatic patients lack fresh CTL activity ex vivo, but this CTL activity could be restimulated by exposure to HIV peptide in vitro (15); in this aspect they resembled typical memory T cell precursors, as seen in the LCMV system. However, Appay et al. correlated low levels of cytolytic activity in HIV-specific tetramer-positive cells with low levels of perforin (16). These cells displayed activation markers like CD69 and were still capable of synthesizing intracellular cytokines. It could be argued that antigen excess had simply stimulated the T cells to discharge their perforin, but these HIV-specific T cells remained perforin deficient even in patients whose antigen load was depressed with antiviral therapy (16). Of note is that even after cultivation in vitro, the HIV-specific but not the CMV-specific T cells from the same patient remained poorly cytolytic and low in perforin. The authors suggested that there may be an impaired maturation of these effector cells during the HIV infection, rather than a subsequent anergization of mature T cells (16).

Both MHC dimers and MHC tetramers have been used to examine HTLV-specific T cells from infected patients, and the antigen-specific T cells from different patients varied greatly in their ability to produce cytokines (17). There also was considerable variation in the ability of in vitro–cultured tetramer-positive clones to secrete cytokines and mediate cytotoxicity (18). Hepatitis C virus–specific tetramer-positive T cells isolated from viremic patients also had deficiencies in their cytokine production and were referred to as "stunned" T cells by one group (19); functionality was restored to the T cells after virus was cleared in vivo.

It therefore seems that a product of persistent infections is T cell dysfunction that can occur in various forms, perhaps affecting both the differentiation of T cells into effectors and the dedifferentiation of effectors into less active states. The same may be true with T cell responses to tumors, which provide another form of continuous antigenic stimulus. Only a low percentage of melanoma-specific tetramer-positive T cells isolated from human peripheral blood-mediated cytotoxicity or secreted cytokines in vitro, whereas EBV-specific T cells from the same patients functioned relatively normally (20), even though some heterogeneity in function of EBV-specific T cells has been observed during EBV infections (21). One unique observation relevant to T cell dysfunction in the melanoma system is that some of the antigen-specific T cells express negatively signaling NK cell receptors (NKRs), such as CD94/NKG2A; antibody to CD94 elevated the killing mediated by tetramer-positive cells coexpressing that NKR (22).

Functional T Cells That Do Not React with Tetramers or Dimers. Although it is now well established that some tetramer-positive T cells lack cytotoxic or cytokine-producing activities, it has also recently been shown that cytotoxic or cytokine-producing T cells may not stain with tetramers. Functional antigen-specific T cells may express this phenotype if their TCRs are significantly downmodulated as a consequence of antigenic exposure. In this issue, Moser et al. document that many tetramer-negative T cells isolated from mice acutely infected with polyomavirus (PyV) are cytotoxic and secrete IFN-γ in response to a PyV peptide (23). Using mAb specific to the TCR β chain, they show that the TCR is downmodulated in the functional, tetramer-negative T cells, providing some of the first clear evidence of receptor downmodulation in a nontransgenic T cell population in vivo.

However, there may be additional reasons for the poor reactivity of T cells with tetramers or MHC dimers. T cells from influenza virus–infected mice transgenic for an influenza virus protein will make an effective peptide-specific CTL and IFN-γ response to a peptide encoded by the transgene, but those T cells react only weakly with MHC tetramers (24). In that case, one would presume either that the presence of the transgene selected for a low avidity T cell population or else that the continued presence of the transgene altered the ability of the influenza-specific TCR to react with tetramers. An interesting study with influenza virus–infected nontransgenic mice by Spencer and Braciale showed that a Flu-specific response to a subdominant epitope gave rise to IFN-γ–producing CTLs that did not stain with tetramers (25). After continued peptide stimulation in vitro, these tetramer-negative Flu-specific T cells acquired an ability to react with the tetramers. The subdominant epitope had some homology to an endogenous mouse peptide sequence, leading the authors to hypothesize that exposure to an endogenous peptide may have caused a redistribution of T cell receptors such that they did not interact appropriately with MHC tetramers. However, after a prolonged period of activation, these cells may have realigned their receptors to make them reactive with tetramers. An explanation for this result may come from recent studies by Fahley et al., who showed, using MHC dimers, that under conditions where changes in total TCR expression were minimal, the activation of a cell can lead to a 30-fold increase in the binding of MHC dimers, but not monomers (26). Adding cholesterol to naive transgenic T cells enhanced their ability to bind dimers, and inhibitors of cholesterol reduced the ability of activated T cells to bind

dimers. This may indicate that the TCR may assemble into lipid raft-like structures that greatly affect their ability to bind MHC dimers or tetramers.

T Cell Responses in Newborn Mice and in Mice with Restricted TCR Repertoires. The age of the host and the availability of the T cell repertoire may greatly influence the outcome of a T cell response. For instance, inoculation of mice with relatively low doses of LCMV within 24 h of birth can give rise to a life-long persistent infection with little evidence of any virus-specific CTL activity (1). Immunization of newborn mice can result in tolerance, and until recently it was questioned whether newborn mice would make T cell responses at all (27). Moser et al. show here that PyV-infection of neonatal C3H/HeN mice, which are genetically resistant to PyV-induced tumor formation, can generate nearly as high a tetramer-specific T cell response to PyV as adult mice; although the kinetics of the response lag in the newborns, these T cells clear the virus and leave the host with a very high frequency of PyV-specific memory T cells, reflective of the T cell clonal burst size (23). Thus, newborn animals can make vigorous CTL responses and clear virus, provided that the viral load does not become too overwhelming, as it probably does during LCMV infections of newborns. A different outcome with PyV was noted in CBA/J mice, which are genetically susceptible to PyV-induced tumor formation. As a consequence of thymic expression of an endogenous mammary tumor virus superantigen, CBA/J mice have part of their T cell repertoire deleted, including T cells needed to control PyV infection (28, 29). These mice clear virus more slowly and develop tumors in response to PyV. Nevertheless, even with this more restricted T cell repertoire, a PyV tetramer-specific T cell response was mounted in newborn CBA/J mice but at levels about one-fourth that of the resistant mice with the more diverse T cell repertoire (23). Of more significance were the qualitative differences between these responses. The viral load remained high in the sensitive mice and their T cells were partially anergic in that they failed to mediate CTL activity. These partially anergic PyV-specific T cells could synthesize IFN-γ and, unlike the HIV-specific T cells (16), contained normal levels of perforin; cytotoxicity could be restored after stimulation in culture for several days (unlike chronic HIV but like acute SIV infections; references 14 and 16). The mechanism for this cytolytic dysfunction is unclear but was associated with high levels of antigen load. Thus, dysfunctional tetramer-positive T cells are found in mice that poorly resist PyV-induced tumor formation, just as they are found in human melanomas (20, 22).

Concluding Comments. New techniques for identifying antigen-specific T cells have shown that they can be very heterogeneous in the expression of antigenic markers, including putative activation/memory markers like CD45RA and CD45R0 (16, 30). Such dramatic heterogeneity is also seen in regards to their functionality. There appear to be several types of dysfunctional antigen-specific T cells that can occur, particularly in the presence of high antigen load: cytokine-producing, poorly cytotoxic cells low in perforin (16), cytokine-producing poorly cytotoxic cells high in perforin (23), poorly cytotoxic cells that do not produce cytokines (10, 14), cytokine-producing cells not readily cytotoxic (including most memory cells before activation [2, 4, 15]), cells in which this anergy can be restored after culture in vitro with antigen and/or IL-2 (14, 15, 23), cells in which these deficient functions can not be restored (16), and cells that have disappeared altogether and have presumably undergone apoptosis (6–8, 10, 14).

What is not clear is whether T cell dysfunction is a consequence of functional arrest at different stages along a linear differentiation pathway or whether a cell may dysfunctionally differentiate in either of several directions. Numerous studies have correlated T cell dysfunction with strength of TCR stimulation and exposure to modulating cytokines, hormones, and oxidative metabolites, so it is possible that, depending on the relative exposure to these signals, a T cell may become more defective in one function (such as cytotoxicity) than another (such as cytokine production). What is clear from virtually all of these systems examined is that the anergization process is very selective to T cells specific for distinct antigens. This argues against a generalized global immune suppression and supports the concept that at least some types of anergization are driven through TCR-mediated signaling.

New studies have identified functional T cells that do not react with MHC dimers or tetramers as a consequence of low avidity due to clonal deletion of high avidity T cells by self (or transgenically expressed) antigens (24, 25), as a consequence of a delay in the maturation of TCR into a more complex organization on the cell membrane, perhaps in association with lipid rafts (24, 26), or as a consequence of receptor downmodulation under conditions of antigen excess (23). We are left with the conclusion that defining T cells by only one parameter of antigenic display or biological function is not sufficient to get an understanding of what is really happening with T cell responses in vivo.

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