*Commentary*

# The Critical Need for CD4 Help in Maintaining Effective Cytotoxic T Lymphocyte Responses

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For many viral infections, resolution of illness is associated with long-term host control of viremia rather than viral eradication. An example is EBV infection, and the fact that host immune suppression is associated with EBV-induced disease suggests that it is the immune system that is holding the virus in check (1, 2). Other viruses are typically associated with progressive uncontrolled disease, and represent an expanding global problem. Over 30 million persons are estimated to be infected with HIV-1 (3), and HCV infection involves 4–6% of the population in certain geographic regions of the world (4). Understanding the correlates of immune protection against such progressive viral infections and the development of effective vaccines thus has great urgency.

Data from both human studies and murine models suggest that CTLs are an important host defense against viruses. CTLs recognize viral peptides that are processed intracellularly and presented at the cell surface as a trimolecular complex with β2-microglobulin and HLA class I. The fact that infected cells can be lysed before the production of progeny virions is indicative of the potency of these cells, at least under idealized laboratory conditions (5). In addition to lysis, recognition of infected cells through the epitope-specific TCR also leads to release of soluble antiviral factors (6). In HBV infection, both TNF-α and IFN-γ have been shown to lead to clearance of virus from infected hepatocytes in a transgenic mouse model (7, 8). In HIV-1 infection, CTL activation by cognate epitope leads to secretion of macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES (9, 10). These antiviral chemokines are localized within cytotoxic granules as a complex with sulfated proteoglycans, and are coordinately secreted with granzymes and perforin, thus exposing the microenvironment of the infected cell with antiviral factors that may serve to inhibit progeny that have already been produced (10).

The recent introduction of more sensitive quantitation methods has provided increased evidence of the critical antiviral role of CTLs (for review see reference 11). Direct flow cytometric visualization of CTLs using tetrameric complexes consisting of HLA class I, β2-microglobulin, and epitopic peptide shows that acute viral infections cause massive CTL expansions that are associated with control of viremia. In acute lymphocytic choriomeningitis virus (LCMV) infection, tetramer staining has revealed that >50% of splenic CD8 cells may be LCMV specific (12), and these high levels of CTLs have been confirmed using functional assays for IFN-γ production by Elispot (12, 13). Similar large expansions of CTLs have been observed in acute human EBV infection where up to 44% of peripheral blood CD8 cells are EBV-specific, class I–restricted CTLs (14). Particularly important insights into the antiviral potential of CTLs in the chronic phase of human viral infection were recently provided by Ogg et al., who showed a negative correlation between tetramer positive, HLA A*0201-restricted CTLs, and control of HIV-1 viremia in untreated infected patients (15).

The above studies demonstrate that CTLs are capable of massive expansion in vivo and are associated with persistent control of viremia. However, CTLs are clearly not effective in some viral infections. Chronic HIV-1 infection is associated with progressive loss of CTLs (16, 17), and chronic HCV infection is associated with low magnitude CTL responses (18, 19). In both of these infections, viremia is uncontrolled. It is thus important to determine what factors control the magnitude and persistence of CTLs, which seem to be critical for efficient control of viremia. An important paper published in this issue by Zajac et al. (20) confirms an essential role for virus-specific T helper cells in maintaining CTL function, and provides new evidence that CTL responses may be silenced in vivo, particularly in situations in which CD4 help is deficient.

The notion that CD4 T helper cells are critical for maintenance of CTLs and chronic control of viremia is not new. The majority of published data come from murine studies of specific strains of LCMV. Although not necessary for the induction of primary CTL responses (21, 22), CD4 help is necessary for maintenance of CTL function during the chronic phase of certain LCMV infections. In particular, under conditions of high dose infection or infection with rapidly replicating and disseminating LCMV strains, CD4 cells are required to prevent exhaustion of CTLs. CD4 cells are not required to clear the less virulent Armstrong strain of virus, which is cleared by CTLs within 8–10 d in the presence or absence of CD4 cells (21, 23). The situation is quite different for the more virulent LCMV strains such as clone 13 and the macrophage-tropic LCMV clone t1B. These viruses, which share 99.8% homology

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with the parental Armstrong strain, exhibit 10–50-fold enhanced replication, and even transient CD4 depletion at the time of infection leads to complete loss of functional CTLs and persistent viremia (23). Likewise, in CD4 knockout mice, infection with a high dose of the viscerolymphotropic LCMV strain WE leads to initial induction of CTLs, but in the absence of CD4 help these CTLs disappear, and chronic infection ensues (24). When $CD4^{-/-}$ mice are infected with the faster replicating LCMV strain DOCILE, even low dose infection leads to abrogation of virus-specific CTL responses and viral persistence (24). A critical role for CD4 cells has also been shown after immunization, in that immunization of mice deficient in CD4 cells leads to less efficient protection from subsequent viral challenge (25). Progressive loss of CTLs in the absence of adequate helper cell function has also been demonstrated for murine γ-herpesvirus (26) and Friend virus infections (27), and has been suggested to occur in humans after adoptive transfer of CMV-specific CTLs (28). Although these studies indicate a link between CD4 help and the magnitude and persistence of CTLs, the mechanisms accounting for this have been largely unclear.

New data presented by Zajac et al. (20) now provide insights into how CD4 deficiency may impair CTL responses, and like other recent advances in understanding of CTLs these data have been facilitated by the direct visualization of CTL using peptide–MHC tetramers (29). A critical finding of this paper—which would have been missed had tetramers not been available—is that antiviral CTLs can persist in vivo in a nonfunctional state, and that this silenced phenotype is more pronounced under conditions of CD4 T cell deficiency. Moreover, epitope specificity and antigen persistence appear to influence whether CTL responses are rendered persistent and nonfunctional or deleted.

Zajac et al. established chronic LCMV infections with the rapidly replicating and widely disseminating LCMV clones 13 and t1B in C57BL/6 mice, and compared these to infections with the weaker Armstrong strain. CTL responses to the dominant class I–restricted envelope epitope (GP33–41) and the nucleoprotein epitope (NP396–404) were quantitated by stimulated CTL assays, limiting dilution precursor frequency assays, ELISPOT for cytokine producing cells, and by direct visualization of epitope-specific CTLs with tetramers. As previously demonstrated, LCMV infection with the less virulent Armstrong strain was associated with viral clearance in $CD4^{+/+}$ mice (22, 23). New analyses using MHC class I tetramers containing epitopic peptides showed that immune mice chronically maintained >2% of CD8 cells specific for GP33 and >5% specific for NP396, and 100% of these cells maintained effector function as evidenced by IFN-γ production. The outcome was similar in $CD4^{-/-}$ mice infected with this less virulent strain, which is consistent with previous studies (24).

In $CD4^{+/+}$ mice infected with the more rapidly replicating and widely disseminating LCMV clones 13 or t1b, viremia was likewise cleared and persistent infection was limited to the kidney. This immune control was associated with strong CTL responses to the GP33 and NP396 epitopes, as evidenced by lysis of infected cells by CD8 cells, elaboration of IFN-γ, and a high level of staining with tetramers. However, tetramer analysis of the $CD4^{+/+}$ mice revealed a selective peripheral deletion of NP396-specific CTLs, but maintenance of GP33-specific CTLs. These cells appeared sufficient to control viremia, eliminating virus from most of the tissues. Only 10–20% of these GP33-specific CTLs remained functional during the chronic phase of infection, again as evidenced by lysis of infected cells and production of IFN-γ. Thus, in the presence of adequate helper cell function, CTL responses to one epitope were deleted in the early stages of infection, whereas CTL responses to a second dominant epitope persisted, but a large fraction were in a nonfunctional state.

The outcome of infection with the more virulent LCMV strains in $CD4^{-/-}$ mice was quite different. In these mice, no functional CTLs could be detected in the chronic phase of infection, and viremia was not controlled. However, tetramer studies showed the initial induction of both GP33- and NP396-specific CTLs, similar in number to those induced in $CD4^{+/+}$ mice, but these CTLs were unable to lyse cells or produce IFN-γ. Despite being nonfunctional, the NP396-specific CTLs were peripherally deleted, with similar kinetics to that of $CD4^{+/+}$ mice. In contrast, the tetramer studies showed that the nonfunctional GP33-specific CTLs persisted throughout the chronic phase of infection in numbers comparable to those seen after infection of $CD4^{+/+}$ mice.

What is the explanation for the silenced phenotype of these CTL? They are turning over in vivo as demonstrated by BrdU labeling. Upregulation of CD69 in the majority of GP33-specific cells indicated recent and continuous exposure to antigen. However, only a small fraction of cells (16%) were capable of eliciting IFN-γ after PMA stimulation, and only a small fraction (15%) were turning over in vivo despite high levels of circulating antigen. There are parallels in the literature to describe T cells with hierarchical responses to different antigen concentrations. Levels of TCR occupancy trigger different biological responses such as cytotoxicity (where even a single peptide–MHC complex may be sufficient to trigger a CTL response; reference 30), IFN-γ release, and IL-2 production (31, 32). The absence of appropriate costimulatory molecules can also markedly increase the number of TCRs that need to be engaged to trigger a T cell response to an agonist peptide (33, 34). Itoh and Germain found that clonal populations of CD4 cells rendered anergic by an antagonist peptide had cytokine profiles resembling those of cells exposed to low levels of antigen or deprived of CD28 costimulation (35). It is possible that sustained TCR signaling from high levels of antigen in the absence of appropriate costimulation could explain this functionally inactivated phenotype. Gallimore et al. have observed a correlation between TCR downregulation on CTLs and prolonged contact with antigen, and have shown that this correlates with decreased CTL activity in the spleen of LCMV-infected mice (36). Their studies

also indicate that with high dose LCMV infection with the WE strain, GP33-specific CTLs are induced, but only a fraction of these make IFN-γ, and are consistent with the findings of Zajac et al. in showing that an anergic phase of CTLs exists between CTL induction and depletion (36).

The nonfunctional CD8 responses were seen in the context of rapidly replicating and widely disseminating LCMV strains, suggesting that infection and dysfunction of APCs may have been involved, preventing effector cells from receiving appropriate costimulation. Borrow et al. demonstrated that interdigitating dendritic cells in the spleen are infectable by LCMV clone 13 and are then targets for virus-specific CTLs (37). The recent work demonstrating that activated CD4 cells are able to "condition" dendritic cells through CD40–CD40L interactions raises the possibility that in the absence of sufficient helper activity dendritic cells may not provide sufficient costimulatory signals to CD8 cells, thus disrupting their normal functions (38–40).

Do these findings help to shed light on persistent uncontrolled human viral infections? The best studied chronic human viral infection is HIV-1, and there are some striking parallels with the LCMV model, both in terms of loss of CTL function and deficiency of T helper cell responses. Numerous studies have verified that progressive infection is associated with loss of CTL responses (16, 17). Our own data indicate that CTLs persist in progressive infection but are incapable of in vivo expansion (Hay, C.M., D.J. Ruhl, N.O. Basgoz, C.C. Wilson, J.M. Billingsley, M.P. DePasquale, R.T. D'Aquila, and B.D. Walker, manuscript submitted). This is certainly consistent with a lack of adequate helper cell function. The most glaring defect in the immune repertoire in HIV-1 infection is the lack of virus-specific T helper cell responses. However, recent studies show that HIV-1 is capable of inducing T helper cell responses, and that the magnitude of T helper cell responses to the HIV-1 Gag protein are associated with control of viremia in untreated persons (41). Particularly strong helper responses are observed in persons who are spontaneously maintaining extremely low viral loads and thus appear to be successfully holding the virus in check, but these people are rare. Individuals who successfully control HIV-1 in the absence of antiretroviral therapy also have strong and persistent CTL responses (reference 42 and Kalams, S.A., S.P. Buchbinder, J.M. Billingsley, E.S. Rosenberg, D.S. Colbert, N.G. Jones, A.K. Shea, A.K. Trocha, G.S. Ogg, P.J. Goulder, and B.D. Walker, manuscript submitted).

Since HIV-1 infects activated CD4 cells and kills them by a fas-dependent interaction (43), a possible mechanism of depletion of virus-specific T helper cells is the infection and elimination of these cells by HIV-1. Acute viral infections can induce large numbers of antigen-specific helper cells, with the LCMV model showing that as many as 10% of CD4 cells may be virus specific at the time of acute infection (44). Such activated cells might be selectively infected and eliminated in the earliest stages of acute HIV-1 infection. If this is true, then protection of activated cells by potent antiviral therapy should prevent loss of these responses. In fact, aggressive treatment of persons with acute HIV-1 infection has been associated with the detection of Gag-specific T helper cells (41). In our own studies of acutely infected persons treated before seroconversion, control of viremia with potent combinations of antiviral therapy including a protease inhibitor results in the predictable generation of strong HIV-1 Gag-specific T helper cell responses, analogous to those seen in persons who are spontaneously controlling viremia in the absence of antiviral therapy (41). In contrast, such responses have not been detected in persons treated in the chronic stage of infection (45), which may be consistent with the findings of Zajac et al., who find that even transient depletion of CD4 cells at the onset of LCMV infection results in the life-long lack of virus-specific CD4 cells.

Although treatment during acute HIV-1 infection results in strong virus-specific T helper cells, whether these responses would contribute to effective control of viremia in the absence of ongoing antiviral therapy has not been tested. Other studies of treatment during acute AIDS virus infections in animals suggest that the magnitude of early viremia may have a profound influence on subsequent disease course. Watson et al. examined this issue in a pathogenic HIV-2 infection model in macaques, in which animals were infected and then treated for just 16 wk with a single nucleoside analogue, D4T. All control animals were dead within 6 mo, as is expected in this model, but 5 out of 6 transiently treated animals remained alive and well with control of viremia (46). Other studies of adoptive therapy with neutralizing antibodies also show that lowering of viral load early on is associated with improved outcome (47). Additional studies are needed to determine the immunologic factors associated with improved outcome.

Another important finding by Zajac et al. is that the fate of CTLs is epitope specific. This issue still needs further testing in humans, which should be forthcoming as tetramers to more HLA class I–restricted CTL epitopes are generated. The only published tetramer studies in HIV-1 infection have concentrated on the CTL response to two HLA A*0201–restricted CTL epitopes, one in the p17 Gag protein (p17/77–85, SLYNTVATL), and the other in the RT protein (RT 476–487, ILKEPVHGV). The tetramer studies of HIV-1–infected subjects found that the percentage of tetramer-positive $CD8^+$ T cells negatively correlated with plasma viremia (15). Although the p17 Gag epitope is more frequently recognized, the levels of RT epitope tetramer-positive cells was also negatively correlated with viral burden, and the combination of both tetramers strengthened this negative correlation. The levels of direct cell lysis are less than would be expected given the percentage of tetramer-positive cells, and perhaps this reflects persistence of some nonfunctional cells as observed by Zajac et al. for the GP33-specific CTL. Given reports that particular HLA types are associated with more rapid disease progression (48, 49), it is possible that the tight correlation between HLA A*0201-Gag-specific CTL and viral load will not be seen with all epitopes. The degree to which differences in outcome may be related to silencing of specific CTL responses in vivo, and the relationship between

epitope density and functional inactivation, both require further attention.

Several recent studies relating to murine control of viral infections need to be examined in comparison to the report by Zajac et al. A major question is whether other cells in addition to CD4 and CD8 cells are critical to viral control. Thomsen et al., examining class II–deficient (and therefore lacking functional CD4 cells) mice, found that CTLs do not persist. However, when similar experiments were performed in mice lacking B cells due to a disruption in the membrane exon of the μ chain gene, CTL memory likewise could not be maintained and virus infection was not contained. Antibody-producing B cells have also been shown to be infected and deleted after LCMV infection (50). After infection with low dose LCMV-WE, C57Bl/6 CD4 or B cell knockout mice are able to initially control viral replication, but after 2 mo virus titers rise and CTL responses are exhausted. The control of viral replication can only be restored after adoptive transfer of both $CD4^+$ T cells and B cells (51). These studies would suggest that effector cells are indeed unresponsive in the presence of high levels of antigen, but also suggest that even a restoration of help may not suffice to mediate control of viral replication. Optimal effector function may exist (and persist) only in the presence of optimal levels of APC function, CD4 help, IFNs, and an appropriate initial antigen concentration. If the phenotype of these impotent effector cells is an effect and not the cause of high levels of antigen, then restoration of help may not suffice to restore effector function in the absence of a reduced viral burden. The answer to this question is critical and has important implications for potential immunotherapeutic interventions to prevent virologic relapse in HIV-1–infected subjects on highly active antiretroviral therapy.

These studies provide strong evidence of yet another way in which CTL control of viremia may be subverted in chronic viral infections, namely by silencing of epitope-specific CTL responses, and also show that CD4 T helper cells play a critical role in determining the fate of effector CTLs. It will be important to determine the relative contribution of inactivated CTLs as a mechanism for persistent viral infection in important human pathogens such as HIV-1 and HCV. An important question to be answered is whether some lack of detection of tetramer-positive cells may be due to TCR downregulation. It will also be critical to determine the precise mechanisms by which CD4 cells contribute to CTL persistence, and whether silenced CD8 cells can be returned to functional competence. Identification of methods to restore function to CTLs should be of critical importance for immunotherapeutic strategies to curtail persistent viral infections.

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