The Journal of Immunology

RESEARCH ARTICLE | FEBRUARY 01 2001

High-Avidity CTL Exploit Two Complementary Mechanisms to Provide Better Protection Against Viral Infection Than Low-Avidity CTL FREE

Michael A. Derby; ... et. al

J Immunol (2001) 166 (3): 1690–1697.
https://doi.org/10.4049/jimmunol.166.3.1690

High-Avidity CTL Exploit Two Complementary Mechanisms to Provide Better Protection Against Viral Infection Than Low-Avidity CTL

Michael A. Derby, Martha A. Alexander-Miller,[1] Richard Tse, and Jay A. Berzofsky[2]

Previously, we observed that high-avidity CTL are much more effective in vivo than low-avidity CTL in elimination of infected cells, but the mechanisms behind their superior activity remained unclear. In this study, we identify two complementary mechanisms: 1) high-avidity CTL lyse infected cells earlier in the course of a viral infection by recognizing lower Ag densities than those distinguished by low-avidity CTL and 2) they initiate lysis of target cells more rapidly at any given Ag density. Alternative mechanisms were excluded, including: 1) the possibility that low-avidity CTL might control virus given more time (virus levels remained as high at 6 days following transfer as at 3 days) and 2) that differences in efficacy might be correlated with homing ability. Furthermore, adoptive transfer of high- and low-avidity CTL into SCID mice demonstrated that transfer of a 10-fold greater amount of low-avidity CTL could only partially compensate for their decreased ability to eliminate infected cells. Thus, we conclude that high-avidity CTL exploit two complementary mechanisms that combine to prevent the spread of virus within the animal: earlier recognition of infected cells when little viral protein has been made and more rapid lysis of infected cells. ***The Journal of Immunology,*** **2001, 166: 1690–1697.**

Cytotoxic T lymphocytes have a well-documented role in the elimination of viral infection. These findings form the basis for numerous vaccine constructs as well as the rationale for the in vitro expansion of CTL for adoptive immunotherapy. The latter has shown some promising results in the clearance of CMV (1–3), EBV (4–7), lymphocytic choriomeningitis virus (8), and HIV infections (9–11), as well as in the control of a number of tumors (12–21). To optimize strategies for immune-mediated viral clearance, it is imperative that we better understand the parameters that control the efficacy with which CTL eliminate virus in vivo. Previously, we demonstrated that avidity is a crucial factor in determining the effectiveness of adoptively transferred CTL: only high-avidity CTL were capable of reducing viral titers in vivo 3 days after the transfer of equal numbers of T cells (22). The greater efficacy of high-avidity CTL has also been confirmed in other systems, both viral (23) and tumor (24, 25). The mechanism of such increased clearance, however, remains unknown. It is equally unknown whether low-avidity CTL would be capable of effecting clearance at later time points. To examine the mechanisms behind the relative in vivo efficacy of high- and low-avidity CTL during viral infections, we investigated 1) the ability of high- and low-avidity CTL to recognize and lyse virally infected cells in vitro as a function of the time course of viral infection; 2) the ability of increased numbers of adoptively transferred low-avidity CTL to compensate for their lower efficacy in vivo; 3) the ability of low-avidity CTL to lower viral titer in vivo if allowed more time to recognize and eliminate vaccinia-infected cells, such as 6 days after infection, as opposed to 3 days; 4) the relative kinetics by which high- and low-avidity CTL kill targets; and 5) the ability of high-and low-avidity CTL to home to the site of infection. Taken as a whole, the results from these studies strongly bolster the efficacy of high-avidity CTL in the reduction of viral titers in vivo and suggest that two complementary mechanisms may combine to account for their superior performance through the earlier elimination of infected cells.

Materials and Methods

Mice and cell lines

BALB/c mice were obtained from both The Jackson Laboratory (Bar Harbor, ME) and the Frederick Cancer and Research Development Center (Frederick, MD), while SCID mice (BALB/c background) were obtained from the Frederick Cancer and Research Development Center. P815 is a DBA/2-derived mastocytoma. 15-12RM is a transfected, tumorigenic BALB/c 3T3 cell line that expresses the gp160 protein from HIV-1_{IIIB} as well as the *ras* and *myc* oncogenes (26, 27). 18neo is a similar control cell line transfected only with the neo vector. BSC-1 is an African green monkey kidney epithelial cell line that allows easy recognition of vaccinia plaques.

Peptide

Peptide p18-I10 (I10), the 10-aa (RGPGRAFVTI), immunodominant peptide epitope within HIV-1_{IIIB} gp160 (26, 28), was obtained from Anaspec (San Jose, CA).

IFN-γ ELISA

IFN-γ was measured in culture supernatants 24 or 48 h after CTL activation using a mouse IFN-γ minikit obtained from Endogen (Cambridge, MA).

Recombinant vaccinia virus

vPE16 is a recombinant vaccinia virus that expresses the gp160 protein from the IIIB strain of HIV-1 (29) and was a kind gift from Patricia Earl and Bernard Moss (National Institute of Allergy and Infectious Diseases, Bethesda, MD). vSC8 is a similar control vaccinia construct that does not express gp160 (30). Stock solutions of virus were stored at −70°C; thawed virus was sonicated three times for 20 s at 4°C before making appropriate dilutions. Virus solutions were titered as described below. For infection of target cells in vitro, 1 ml of cell culture medium containing 5×10^7 PFU of vaccinia was added to 1×10^6 P815 cells and then incubated at 37°C until assayed.

Molecular Immunogenetics and Vaccine Research Section, Metabolism Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892

Received for publication July 19, 2000. Accepted for publication November 14, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Current address: Department of Microbiology and Immunology, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157.

[2] Address correspondence and reprint requests to Dr. Jay A. Berzofsky, Molecular Immunogenetics and Vaccine Research Section, Metabolism Branch, National Cancer Institute, National Institutes of Health, 10 Center Drive, Bethesda, MD 20892-1578. E-mail address: berzofsk@helix.nih.gov

0022-1767/01/$02.00

In vivo CTL reconstitution and vaccinia titers

H-2^d SCID mice were inoculated i.v. via the tail vein with varying amounts of CTL in 100 μl to 1 ml of PBS. CTL used in reconstitution experiments had been restimulated 4 or 5 days before. Immediately following CTL administration, 1–5 $\times$ 10^7 PFU of recombinant vaccinia were injected i.p. in 200 μl of PBS. The quantity of virus is critical since excessive viral titers obscure the protection of CTL adoptively transferred into SCID mice. Three or 6 days later, mice were sacrificed and tissues were harvested. Samples were frozen at −70°C until analysis for vaccinia PFU as per Buller and Wallace (31). Briefly, ovaries were frozen and thawed three times, homogenized in 1 ml of PBS, sonicated for 1 min, and spun briefly to obtain a clear supernatant containing virus. Serial dilutions of the supernatant were made in medium containing 2.5% FBS and 1 ml of each supernatant was added per well to monolayers of BSC-1 cells in a six-well plate. After 2 h, 2 ml of additional medium was added. Forty-eight hours later, the cell monolayers were fixed and stained, and the vaccinia plaques were evaluated to ascertain the PFU per ovary.

CTL homing and sectioning of ovarian tissue

CTL were labeled by incubation with 10 μM 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (diIC$_{16}$)[3] (Molecular Probes, Eugene, OR) for 15 min at 37°C or 2.5 μM Cell Tracker Green (Molecular Probes) for 45 min at 37°C before washing in PBS before use. CTL (1 $\times$ 10^7) were injected into the tail vein of SCID mice, followed immediately by i.p. administration of 5 $\times$ 10^7 PFU of vPE16. Twenty-four to 48 h later, the ovaries were removed. If labeled with diIC$_{16}$, they were embedded in OCT, frozen on dry ice, and stored at −70°C before sectioning with a cryostat. Ovaries labeled with Cell Tracker Green were fixed with 4% formaldehyde in PBS and processed for conventional paraffin embedding and sectioning. In either case, sections were analyzed by fluorescent microscopy and CTL were quantified by obtaining the mean ± SD of the total CTL in ×400 high-powered fields of ovary sections from each of three separate experiments. These two methods are complementary: diIC$_{16}$ requires visualization within frozen sections with corresponding suboptimal histology, whereas Cell Tracker Green can be visualized within paraffin sections, but against a high autofluorescence inherent to ovarian tissues.

Generation of CTL lines and clones

CTL lines were generated using either APC pulsed for 2 h with peptide or the addition of free peptide to cocultures of CTL and APC. Initial cultures were started using cells obtained from the spleens of BALB/c mice immunized 1–3 mo previously with vPE16. For lines generated with pulsed APC, 7.5 $\times$ 10^6 responding spleen cells were cocultured with 3.5 $\times$ 10^6 irradiated (3000 rad) stimulating BALB/c splenocytes pulsed with various concentrations (1 $\times$ 10^2, 1 $\times$ 10^{-1}, or 1 $\times$ 10^{-4} μM) of I10 peptide in a 24-well plate containing 2 ml of RPMI 1640 medium supplemented with HEPES, L-glutamine, sodium pyruvate, nonessential amino acids, penicillin, streptomycin, 5 $\times$ 10^{-5} M 2-ME, 10% FCS, and 10% T-Stim (Collaborative Biomedical Products, Bedford, MA). Lines generated with free I10 peptide were prepared similarly, but had 5 $\times$ 10^{-5}–5 $\times$ 10^0 μM free I10 added directly to the cultures. CTL lines were established from primary cultures and were maintained in 24-well plates by weekly restimulation of 3–5 $\times$ 10^5 cells/well in the presence of 5 $\times$ 10^6 irradiated (3000 rad) BALB/c spleen cells either pulsed with the appropriate concentration of I10 peptide or with I10 peptide added directly to the culture. For experiments requiring greater numbers of CTL, 1 $\times$ 10^7 CTL were grown in T75 flasks in the presence of 0.5–1 $\times$ 10^8 irradiated spleen cells in 12.5 ml of medium containing free peptide. CTL clones were established from CTL lines of various avidities by limiting dilution cloning. Individual clones were obtained from plates containing growth in <8 of 96 wells, screened for uniformity of TCRVβ usage, and maintained under conditions appropriate for their avidity.

Avidity

CTL avidity was defined as the negative log of the peptide concentration that resulted in 50% maximal target cell lysis. CTL avidity was also assessed by determining susceptibility of target cell lysis to blockade by 10 μg/ml anti-CD8 (clone 53-6.7; PharMingen, San Diego, CA).

[3] Abbreviations used in this paper: diIC$_{16}$, 1,1′-dihexadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; BATDA, bis(acetoxymethyl)-2,2′:6′,2′-terpyridine-6,6′-dicarboxylate.

^{51}Cr release and europium/bis(acetoxymethyl)2,2′:6′,2′-terpyridine-6,6′-dicarboxylate (BATDA) assays

The ^{51}Cr release assay was conducted as previously described (32). Europium/BATDA assays were performed similarly. Target cells (1 $\times$ 10^6) were either unpulsed or pulsed with an appropriate concentration of peptide in culture medium containing 2 mM probenecid for 2 h at 37°C. A volume of 5 μl of BATDA was added during the last 15 min of labeling. BATDA-loaded APC were washed five times in 12.5 ml of warm culture medium containing 2 mM probenecid, with a 20 min rest between the fourth and fifth washes. CTL were added at a 12.5:1 E:T ratio to the peptide-pulsed, BATDA-loaded, and washed APC and incubated in culture medium that also contained 2 mM probenecid. At one or more time points, from 1 to 4.5 h, 20-μl samples of supernatant were added to 200 μl of europium solution in a 96-well plate. Spontaneous release of BATDA from P815 target cells prevented extension of assays beyond 4.5 h. After shaking for 15 min, free BATDA released into the supernatant by lysis of the target cells could be measured by the time-delayed fluorescence of BATDA-chelated europium in a Wallac Victor^2 (Perkin-Elmer, Gaithersburg, MD). When wells were sampled at only one time point, the specific lysis was calculated identically to that for the ^{51}Cr release assay. Since europium assays allowed the same well to be sequentially sampled to examine the kinetics of lysis, a modification of the formula was used to account for the serial 20-μl reductions in the 200-μl initial well volume after sampling at each time point. The following formula was used to calculate the specific fluorescence of sample *n*:

$$\sum_{1\to n}\left(\left(\left(\frac{fluorescence_n - bkgnd_n}{fluorescence_{MAX} - bkgnd_n}\right) - \left(\frac{fluorescence_{n-1} - bkgnd_{n-1}}{fluorescence_{MAX} - bkgnd_{n-1}}\right)\right) \times \left(\frac{S-(n-1)}{S}\right)\right),$$

where $fluorescence_n$ is the measured europium time-delayed fluorescence at time point *n*; $fluorescence_{MAX}$ is the maximum fluorescence of the targets released by 0.1% Triton X-100; $bkgnd_n$ is the fluorescence due to spontaneous release of BATDA at time point *n*; and *S* is the number of potential samples in the well ($S = 10$, when removing 20-μl samples from a well with an original volume of 200 μl). In those experiments where the avidities of CTL lines were directly compared, a peptide concentration was determined that resulted in a plateau of maximal lysis for all CTL lines and clones by 4.5–6 h, and the CTL lysis of target cells was normalized to 100% of maximal lysis at that Ag density. With I10 peptide, even low-avidity lines or clones showed maximal lysis of target cells pulsed with concentrations >5 μM I10 peptide. Maximal lysis usually varied between 70 and 90% of total APC.

Results

CTL clones retain the properties of their parental CTL lines

CTL lines were generated using either peptide-pulsed APC or by addition of free peptide to the culture medium (22). Regardless of the means used to generate them, CTL lines of a given avidity exhibited similar properties. High-avidity cells could be generated using either low concentrations of peptide pulsed onto APC or by addition of low concentrations of peptide to the culture medium. Very low-avidity cells, on the other hand, were best generated by addition of high concentrations of free peptide to the culture medium; equivalent high Ag densities could not be easily obtained using lines generated with pulsed cells. Since previous work was all done with uncloned CTL lines, for this study CTL clones were generated by limiting dilution from high-avidity, low-avidity, and very low-avidity CTL lines and found to always retain the properties of their parental cell lines (Fig. 1*A*). They exhibited a 3–4 $\log_{10}$ difference in functional avidity measured as peptide concentration necessary to produce either 50% lysis (Table I) or IFN-γ (Fig. 1*B*). Avidity was also confirmed by the sensitivity of Ag-density requirements to anti-CD8 Ab (Ref. 22; data not shown). Since all of the CTL lines and clones make IFN-γ, they are all of a similar Tc1 phenotype.

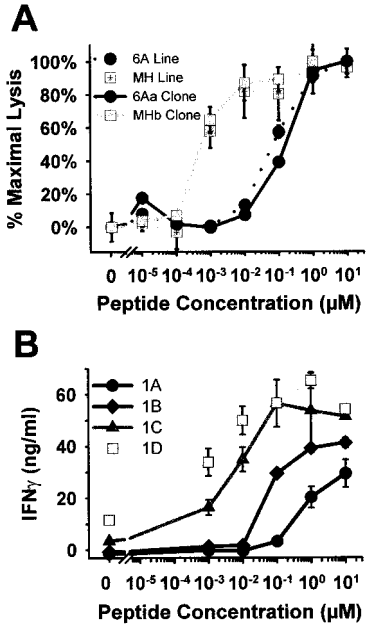

FIGURE 1. *A,* Clones derived from a given CTL line exhibited properties identical to their parental cell lines. CTL lines were added at an E:T ratio of 10:1 to P815 target cells pulsed for 2 h with the indicated concentration of I10 peptide and examined in a ^{51}Cr lytic assay. Target cell lysis is expressed as the mean ± SD of the percentage of the maximal lysis within each CTL line. Clone 6Aa was derived from the 6A CTL line; clone MHb was derived from the MH CTL line. *B,* High- and low-avidity CTL demonstrate at least a 3 $\log_{10}$ difference in the Ag density necessary to stimulate IFN-γ production. High- and low-avidity CTL were added at an E:T ratio of 12.5:1 to P815 target cells pulsed for 2 h with the indicated concentration of I10 peptide. After 48 h of culture, supernatant was examined for IFN-γ production. CTL lines increase in avidity (as determined by target cell lysis; data not shown) from 1A to 1D.

High-avidity CTL recognize endogenously presented Ag on target cells at earlier time points after viral infection

Previously, we demonstrated that high-avidity CTL, but not low-avidity CTL, were effective at reducing viral titers in vivo (22). This preferential reduction occurred despite the ability of both high- and low-avidity CTL to recognize virally infected cells in vitro. We hypothesized that the lower viral titers seen in vivo after adoptive transfer of high-avidity CTL might be due to the ability of these CTL to recognize and lyse target cells earlier in the course of a cellular viral infection. At such times, just as viral components were initially synthesized, the Ag densities would be so low that only the high-avidity CTL would be able to recognize infected cells. Correspondingly, low-avidity CTL, which require a high peptide/MHC determinant density, would be unable to recognize and lyse target cells until later in viral development when higher amounts of peptide-MHC complexes would be displayed at the cell surface and viral progeny were already likely to have been assembled. The hypothesis was examined by contrasting the times at which high-and low-avidity CTL were first able to recognize and lyse target cells infected with vPE16, a recombinant vaccinia virus containing HIV-1_{IIIB} gp160. Lytic assays were initiated by addition of CTL to P815 target cells at various times after infection of the target cells with vPE16 virus, and the total target cell lysis was assessed after a 4-h incubation. As shown in Fig. 2, high-avidity CTL recognized vaccinia-infected targets as early as 2–4 h after infection with vPE16. In contrast, low-avidity CTL could recognize targets only at later time points. Furthermore, even though both high- and low-avidity CTL were able to similarly recognize and lyse peptide-pulsed target cells presenting very high Ag densities (indicating a similar lytic activity if Ag density became sufficiently high), high-avidity CTL were much more effective in killing virus-infected cells up to 22 h after the onset of infection (Fig. 2). In particular, the slopes of the kinetic curves for target cell lysis are substantially different during the first 8 h. These results support the hypothesis that high-avidity CTL recognize virus-infected cells earlier in the time course of infection than do low-avidity CTL, and thus prevent an increase in viral titer by killing infected cells before the assembly and release of new virus.

Table I. *Pulsed peptide concentration resulting in 50% lysis of target cell (functional avidity)*

CTL Clone	Peptide Concentration (μM)
6Aa (very low avidity)	$1.1 \times 10^{+0} \pm 4.3 \times 10^{-1}$
MLd (low avidity)	$3.4 \times 10^{-1} \pm 2.7 \times 10^{-1}$
1Da (moderate avidity)	$5.6 \times 10^{-3} \pm 8.6 \times 10^{-4}$
MHb (high avidity)	$1.6 \times 10^{-3} \pm 3.9 \times 10^{-4}$
1Ea (high avidity)	$1.2 \times 10^{-4} \pm 3.6 \times 10^{-5}$

To further characterize the ability of CTL of high-or low-avidity to recognize and lyse target cells presenting low Ag densities, we characterized the ability of high-, low-, or very low-avidity CTL clones to lyse either 1) 15-12RM cells, a cell line transfected with $gp160_{IIIB}$ that endogenously presents low levels of I10 (although gp160 mRNA could be detected, no gp160 protein was detected by Western blot (26, 27)); 2) 18neo cells, a matched control cell not containing gp160; or 3) 18neo cells pulsed with 50 μM I10 to produce a high Ag density (Fig. 3). All CTL clones could recognize I10-pulsed 18neo control cells, but only the high-avidity clone was able to recognize and lyse both the 15-12RM cells and the I10-pulsed 18neo control cells equally well. The low-avidity clone recognized the 15-12RM cells with a lowered efficacy, whereas the very low-avidity 6Aa clone did not lyse the 15-12RM cells at all, even though it was capable of recognizing the 18neo cells presenting high peptide-Ag densities. These results confirm that high-avidity CTL can recognize Ag presented endogenously at densities not seen by lower avidity CTL, and further support the hypothesis that high-avidity CTL are capable of recognizing and killing cells earlier in the course of viral infection.

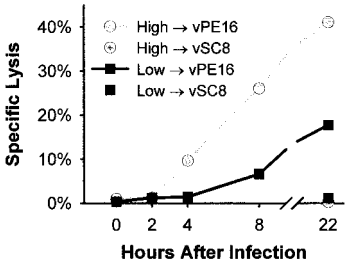

FIGURE 2. High-avidity CTL recognize virus-infected target cells earlier and more effectively than low-avidity CTL. High- or low-avidity CTL were added to target cells at various times after infection of the targets with vPE16 recombinant vaccinia virus and then target cell lysis was determined after a 4-h incubation. High-avidity CTL showed measurable lysis of infected targets in assays initiated as early as 2–4 h after target infection, whereas low-avidity CTL did not show measurable lysis until assays initiated 8 h after viral infection. Target cells infected with the control vaccina virus vSC8, which did not contain gp160, showed only background lysis, whereas both high- and low-avidity CTL showed equivalent lysis of target cells pulsed to high Ag density with 10 μM peptide (63 and 57%, respectively), indicating that the overall activity of the CTL lines was matched in the experiment.

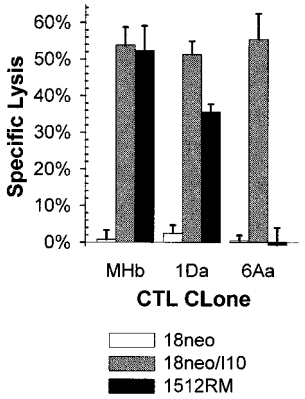

FIGURE 3. High- and low-avidity CTL clones are able to recognize and lyse cells endogenously producing low levels of Ag, whereas very low-avidity cells can recognize only the high Ag densities on peptide-pulsed control cells. MHb high-avidity, 1Da low-avidity, or 6Aa very low-avidity clonal CTL were added to 15-12RM cells, which endogenously produce low levels of HIV-1_{IIIB} gp160, or to a control cell line, 18neo, which was either pulsed or unpulsed with 50 μM I10 peptide at an E:T ratio of 12.5:1. Values are expressed as mean ± SD.

High-avidity CTL initiate lysis of peptide-pulsed target cells earlier than low-avidity CTL

The previous results indicated that high-avidity CTL could recognize the low Ag densities resulting from endogenous vPE16 infection earlier than low-avidity CTL, but since these results were obtained using ^{51}Cr lytic assays that represented the total lysis present after ≥4 h of incubation, they did not address differences in the rate of lysis. It remained possible that high-avidity CTL not only recognized lower Ag densities, but also killed target cells at a higher constant rate. The kinetics of lysis of targets pulsed with a fixed amount of peptide by CTL of varying avidity is distinct from the kinetics by which infected targets become susceptible to lysis as increasing levels of viral protein are made, as was described in Fig. 2. To examine this question, we used the Europium lytic assay to compare the ability of four clones of differing avidity to lyse target cells presenting high Ag density. As seen in Fig. 4*A*, when targets were pulsed with 10 μM I10 peptide, a concentration allowing lysis by all of the CTL, high-avidity CTL began to lyse the target cells immediately, but lower avidity CTL revealed a delay in the onset of their lytic activity. Once lysis began, however, the lower avidity CTL killed target cells at a rate similar to that of the higher avidity CTL. Due to the relationship between CTL avidity and Ag density, we also examined the lytic kinetics of high-avidity CTL over a range of Ag densities. Fig. 4*B* shows that as the Ag density decreased to a range below that detectable by the lower avidity CTL (1×10^{-3} μM), the high-avidity CTL showed an increasing delay in their activation. As seen above with the low-avidity CTL, however, once lysis was initiated, the rates of lysis were similar. Thus, high-avidity CTL initiate target cell lysis more quickly at Ag densities at which both high- and low-avidity CTL can respond, but they also initiate target cell lysis, albeit not as quickly, at densities at which low-avidity CTL do not function at all. Therefore, a second mechanism by which CTL may more effectively reduce viral burden is their ability to initiate lysis earlier at a given level of target Ag expression and thus kill more infected target cells in a shorter time.

In vivo, high-avidity CTL reduce viral burden at least 10-fold more effectively than low-avidity CTL

Since we had previously demonstrated that low-avidity CTL lines are less effective in reducing viral burden in vivo than equivalent

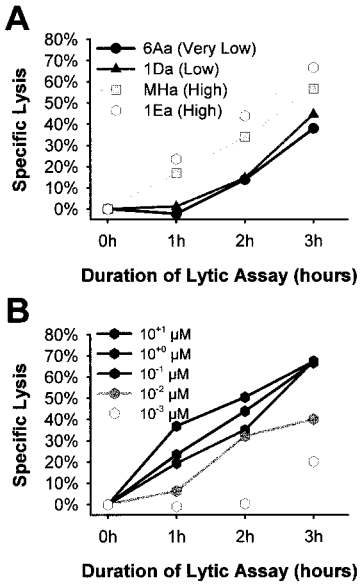

FIGURE 4. High-avidity CTL initiate an earlier and more rapid lysis than low-avidity CTL. *A*, 6Aa very low-avidity, 1Da low-avidity, and MHa and 1Ea high-avidity CTL clones were added to P815 targets pulsed with 10 μM I10 peptide in a europium lytic assay and wells were then sampled after 1, 2, or 3 h of incubation. The results are typical of more than four experiments. *B*, 1Ea high-avidity CTL were added to targets pulsed with various Ag densities and cell lysis was then assessed at 1, 2, and 3 h after initiation of the assay. Similar results were obtained in at least three other experiments.

numbers of high-avidity CTL (22), but had never quantitatively compared their potency in vivo by titrating the numbers of cells transferred, we asked whether transferring increased numbers of low-avidity cells could compensate for their lower efficacy. As is shown in Fig. 5*A*, increasing the number of low-avidity CTL to 3×10^7, the maximum number of low-avidity CTL reasonably generated for adoptive transfer with our culture system, still produced little reduction in viral titer. On the other hand, we were surprised to find that a 3-fold reduction in the number of high-avidity CTL transferred (from 1×10^7–3.3×10^6) significantly reduced their effectiveness (Fig. 5*A*). We extended these observations by repeating this experiment using high-, low-, and very low-avidity CTL clones. As seen in the earlier experiment using CTL lines, the high-avidity CTL clone showed a sharp decrease in effectiveness with a small drop in the number of cells transferred (Fig. 5*B*). CTL from the low-avidity 1 Da clone, which corresponded most closely in avidity to the low-avidity line used in the first experiment, showed a small decrease in viral titer at the highest concentration transferred. The lowest avidity clone, on the other hand, showed no reduction in viral titer at any concentration examined. Since effective clearance of vaccinia virus in vivo has been ascribed to IFN-γ (33), we examined the levels of IFN-γ produced by CTL clones and lines of various avidities. Although the Ag densities required for cytokine production reflected the CTL avidity (Fig. 1*B* and Refs. 34 and 35), there were no consistent associations of IFN-γ production with CTL avidity because, in general, IFN-γ production at high Ag density was roughly equivalent (Table II). In summary, either high-avidity clones or CTL lines produced a response curve in vivo shifted more than a log to the left of low-avidity clones or CTL lines, indicating more than a 10-fold greater efficiency in reduction of viral titers in vivo.

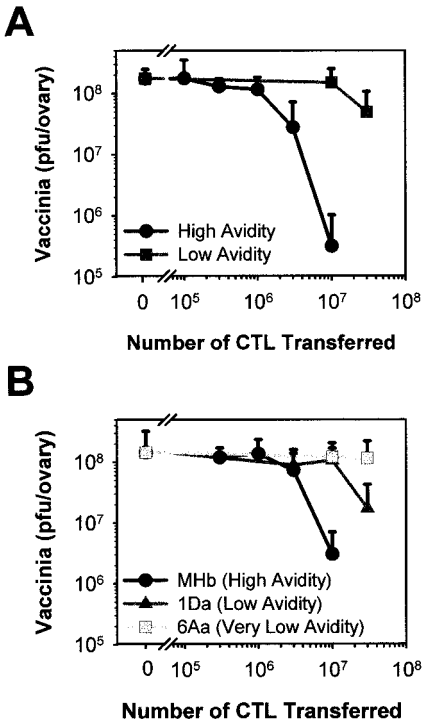

FIGURE 5. Only high-avidity CTL lines or clones can provide effective protection against high viral titers following viral infection, and that protection is very dependent upon the number of CTL transferred. *A,* High- and low-avidity CTL lines were adoptively transferred i.v. into BALB/c SCID mice simultaneously infected i.p. with vPE16 recombinant vaccinia virus expressing HIV-1_{IIIB} gp160. Three days later, ovaries were harvested and the viral titer was measured. Values are expressed as mean ± SD. *B,* Between 1×10^6 and 3.3×10^7 high-, low-, or very low-avidity clonal CTL were injected i.v. into mice simultaneously infected i.p. with vPE16. Results were similar to those obtained for CTL lines.

Kinetics of viral clearance by high- and low-avidity CTL in vivo

In the previous experiments in which low-avidity CTL were shown to be ineffective for viral clearance, only a single time point (3 days after infection) was analyzed. Given that low-avidity CTL showed a delayed and slower lytic rate in vitro and transferring larger numbers of low-avidity CTL increased their ability to lower viral titer (see Fig. 5), the possibility remained that low-avidity CTL were capable of eliminating virus in vivo but did so with slower kinetics. To determine whether this was indeed the case, we compared the viral burden 3 and 6 days after transfer of high- and low-avidity CTL. Fig. 6 shows that these low-avidity CTL remain incapable of reducing viral titers even when given a longer time to act. Thus, the lack of clearance is an absolute and not a kinetic difference between high- and low-avidity CTL in vivo.

Both high- and low-avidity CTL are capable of homing to the ovary

The increased effectiveness of high-avidity CTL to reduce viral titers after infection could potentially be due either to their ability to home to the site of infection earlier than low-avidity CTL or to their ability to lyse infected cells earlier during the course of infection. To distinguish between these alternatives, we examined whether high-avidity CTL home to the site of infection earlier than low-avidity CTL. Following the same procedure employed for adoptive transfer experiments, 1×10^7 CTL labeled with either lipid-permeable diIC_{16} or cytoplasmic Cell Tracker Green were injected into the tail vein of SCID mice which simultaneously received 5×10^7 PFU vPE16 i.p. After 24 or 48 h, ovaries were harvested and sectioned to compare the efficiency of homing of both high- and low-avidity CTL. Examination of histological sections showed no significant difference between the numbers of high- or low-avidity CTL that were irregularly distributed throughout the ovary, primarily in the ovarian stroma, by 24 h after infection (Fig. 7). Similar results were seen at 48 h (although increasing inflammation and vesicle formation in control ovaries and those receiving low-avidity CTL made histology more difficult), and when ovary cells were dispersed and examined by flow cytometry (data not shown).

Table II. *IFN-γ production 48 h after activation of independently derived sets of CTL lines with 5 μM peptide-pulsed APC*

CTL Line	IFN-γ (ng/ml)
5A (very low avidity)	60.70 ± 13.42
5C (low avidity)	60.79 ± 6.56
5E (high avidity)	64.13 ± 6.12
6A (very low avidity)	57.84 ± 11.29
6C (low avidity)	64.58 ± 12.47
6E (high avidity)	46.51 ± 9.36

Discussion

The use of in vitro expanded CTL for adoptive immunotherapy is a promising avenue in the treatment of viral disease as well as cancer. Since technical issues limit the number of CTL which can realistically be obtained and transferred, it seems prudent to select and expand those CTL which will be most efficacious in vivo. To achieve this goal, one needs to understand what attributes will promote increased efficacy and how to obtain CTL possessing these characteristics.

Previously, we used peptide-pulsed APC to generate high- and low-avidity CTL lines. High-avidity CTL resulted from expansion using APC pulsed to present low Ag densities, whereas low-avidity CTL resulted from CTL exposed to APC presenting high Ag densities. In an attempt to simplify and expedite the production and maintenance of such lines, we generated a series of lines against various concentrations of free peptide. It was possible to generate equivalent CTL lines by using either APC pulsed with peptide or by using free peptide added to cocultures of CTL and APC, but although all high-avidity CTL shared similar properties, as did

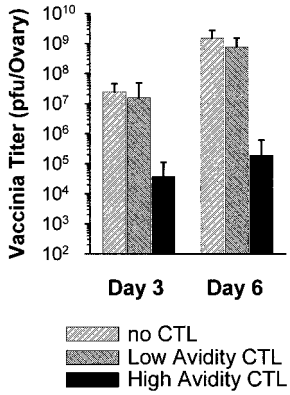

FIGURE 6. Low-avidity CTL are unable to lower the viral titer after a more extended time in vivo. High- and low-avidity CTL lines were adoptively transferred i.v. into BALB/c SCID mice simultaneously infected i.p. with vPE16 recombinant vaccinia virus expressing HIV-1_{IIIB} gp160. Either 3 or 6 days later, ovaries were harvested and the viral titer was determined. Values are expressed as mean ± SD.

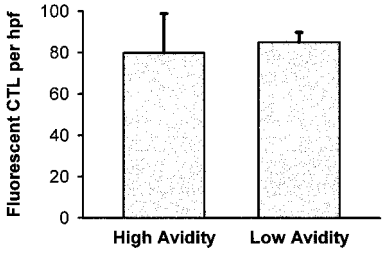

FIGURE 7. High- and low-avidity CTL clones home to the ovary with equal efficacy. High- and low-avidity CTL were labeled in vitro with Cell Tracker Green. CTL were injected i.v. simultaneously with i.p. injection of vPE16 into BALB/c SCID mice. One day later, the mice were killed and the CTL present in ovarian stroma were tallied as the number of fluorescent cells per high-powered field (hpf).

low-avidity CTL, the concentrations of peptide required to generate a given avidity varied between the two approaches. We also isolated clones from CTL lines with a range of avidities to avoid problems inherent with the use of a population of cells that shared a common avidity, but which might differ in their TCR affinity. Despite the potential variability of CTL lines, clones generated from a specific line shared nearly identical avidity with the parental line (Fig. 1*A*).

In our previous study, we demonstrated that a fixed number of high-avidity CTL was much more effective than the same number of low-avidity CTL in reducing viral titers in vivo (22), but we did not compare the CTL quantitatively by titration in vivo. The current study was designed to elucidate the mechanism underlying this increased efficacy. Here, we show that lower avidity CTL remained less capable of reducing viral burden, even when the number of transferred CTL is increased 3-fold or when more time is allowed for clearance. This was perhaps surprising since both high- and low-avidity lines could recognize virally infected targets in vitro (22). We hypothesized that the mechanism underlying the lack of effectiveness in vivo was a result of the amount of viral protein required to obtain the threshold determinant density necessary for recognition by low vs high-avidity CTL and the efficiency by which high-avidity CTL could then eliminate infected cells. Previous investigators have postulated that CTL that can recognize target cells early in infection, when the density of viral peptide-MHC complexes is still low, may be more effective in clearing virus infection than CTL that can kill only targets already loaded with high levels of viral progeny (36–38). In contrast, low-avidity CTL requiring an Ag density so high that it is achieved just before the release of assembled virus from a cell are unlikely to be as effective in the control of viral titers, even though as the infection progressed and viral titers increased, their efficacy would undoubtedly improve. In Fig. 2, we show that high-avidity CTL are more capable than low-avidity CTL of recognizing target cells early in the course of viral infection, as seen by the substantial difference in slopes within the first 8 h.

Further evidence that determinant density controls the recognition of infected cells is provided by the CTL lysis of 15-12RM target cells. 15-12RM cells endogenously present low levels of I10 Ag, as the gp160 protein cannot be detected by Western blot even though the mRNA can be detected (26). Only high-avidity CTL can recognize this cell line as effectively as they recognize control 18neo cells pulsed with peptide. Low-avidity CTL are less effective, and the lowest avidity CTL do not recognize the 15-12RM cells at all, despite being able to recognize and lyse 1) P815 target cells infected with vPE16 recombinant vaccinia virus and 2) control target cells pulsed with high concentrations of peptide Ag as effectively as high-avidity CTL. In vivo, it is not clear whether CTL actually clear virus directly by lysis of infected cells or indirectly through the action of cytokines such as IFN-γ (33). Regardless, since cytokine production and lytic activity are equally correlated with avidity (Refs. 34 and 35 and Fig. 1*B*), either could account for the observed differences in efficacy.

These observations support the hypothesis that the amount of time required for infected cells to present an adequate determinant density is a limiting factor for lysis of infected cells by low-avidity CTL and that the delay incurred with the low-avidity CTL allows for the production of increasing amounts of assembled virus and a perpetuation of the infection. Furthermore, as we observed in Fig. 4, this difference is compounded by the fact that, in contrast to high-avidity CTL, low-avidity CTL exhibit a delay in the onset of their lytic activity, presumably due to inherent differences in the efficiency of TCR signaling. Once initiated, the rates of lysis seem equivalent, but any delay in the onset of lysis only provides more time for viral proliferation and contributes to the reduced effectiveness of low-avidity CTL in combating viral infections. Further evidence indicating that this delay is inherent in the TCR signaling efficiency appears at very low Ag densities when high-avidity CTL also show a delay in the onset of lytic activity. Taken together, these results indicate that two complementary mechanisms combine to account for the greater ability of high-avidity CTL to prevent an increase in viral titer by killing infected cells before the assembly and release of new virus: 1) high-avidity CTL recognize and kill virus-infected cells earlier in the course of infection at very low Ag densities and 2) high-avidity CTL have more rapid initiation of lysis and thus a more rapid elimination of infected targets before substantial production and release of functional virus. This latter mechanism may reflect more efficient TCR signaling, as suggested by differences in functional avidity in transgenic cells with the identical TCR, or in cells in which the TCR affinity did not correlate completely with functional avidity.[4,5] Although both mechanisms may represent corollaries that derive from the same fundamental property, they actually represent functionally different aspects operating independently in time and complementing each other to produce the greater efficacy of high-avidity CTL. One corresponds to the generation of Ag density during virus replication, whereas the other reflects the rate at which CTL are able to recognize that Ag density during TCR signaling. Thus, in vivo, enhanced recognition of low levels of viral Ag and superior TCR kinetics are likely to be the determining factors in the differential ability of high-avidity CTL to reduce viral titers.

The lag in onset of lysis by low- vs high-avidity CTL, or by high-avidity CTL at very low Ag densities, is of interest in its own right. One of the factors contributing to CTL avidity is the affinity of the TCR. One might speculate that the onset of lysis requires some threshold number of TCR molecules to be engaged or aggregated (39) or to be serially triggered (40, 41). In either case, since low-affinity TCRs would be expected to have a much shorter average dwell time for engagement of the peptide-MHC complex than that of high-affinity TCRs, the probability that an encounter between a TCR and its ligand would last long enough to result in a productive signal would be lower for low-avidity CTL. Therefore, it might take longer to achieve the threshold necessary for activation of the CTL. Similarly, even for high-affinity TCRs, if the density of relevant peptide-MHC on the target cell was too

[4] M. A. Derby, J. Wang, D. H. Margulies, and J. A. Berzofsky. Two intermediate avidity CTL clones with a disparity between functional avidity and MHC tetramer staining. *Submitted for publication.*

[5] A. G. Cawthon, H. Lu, and M. A. Alexander-Miller. Sensitivity to TCR engagement as a mechanism to control CTL avidity. *Submitted for publication.*

sparse, it might take longer to accumulate sufficient interactions to reach this threshold.

Our previous work demonstrated that adoptively transferred high-avidity CTL could effectively prevent the accumulation of high vaccinia titers in the ovary, the principle site of vaccinia proliferation in female mice (Ref. 22 and data not shown), whereas low-avidity CTL were generally ineffective. To explore other possible mechanisms that might also contribute to reduction in viral load, in this work we examine whether low-avidity CTL would be more effective either at higher concentrations or after an extended period of interaction with infected cells in vivo. This is particularly relevant, since, in vitro, high-avidity CTL both recognize lower Ag density and initiate lysis more rapidly than low-avidity CTL, and others have shown that time is critical in an effective in vivo response (38). Fig. 5 demonstrates that high-avidity CTL are clearly more effective at reducing viral titers than low-avidity CTL, but, interestingly, viral titer increased rapidly with a small decrease in the number of high-avidity CTL transferred. One explanation for this might be that we used a SCID model system to examine adoptive transfer to isolate the effects of the $CD8^+$ CTL on the reduction of viral titer. SCID mice lack those components of the immune response that might be expected to effectively clear viral particles. Adoptively transferred CTL can reduce the accumulation of new virus, but are unlikely to reduce virus once already produced. Interestingly, when Gallimore et al. (23) transferred CTL into immunologically intact B6 mice, they also found that high-avidity CTL reduced viral titers more than lower avidity CTL, but in their system protection by both high- and low-avidity CTL showed substantial improvements as the number of CTL transferred increased. Viral titers measured in the ovary on day 3 or 6 were heavily influenced by the initial viral burden injected on day 0. If the initial viral burden were sufficiently high, it was impossible to demonstrate a reduction in viral titer on day 3 or 6, even following the transfer of 3×10^7 high-avidity CTL (data not shown). Thus, in this SCID model system, adoptively transferred CTL must recognize infected cells early enough in the course of viral replication that they prevent the accumulation of new virus particles, but if the initial viral burden is too high, their efforts may go unrecognized. Low-avidity CTL would then not be expected to be more effective given increased time in contact with infected cells, since even if they could recognize higher Ag densities at later time points, high viral titers would already have accumulated. The results in Fig. 2, showing that high-avidity CTL are capable of recognizing infected target cells much earlier than low-avidity CTL, as well as those in Fig. 4, showing that low-avidity CTL exhibit a delay in the onset of lysis, argue that by the time low-avidity CTL could recognize infected cells in vivo, viral progeny might already be assembled. Consistent with this prediction, Fig. 6 shows that, after adoptive transfer and infection of mice with vPE16, low-avidity CTL were no more effective at reducing viral titers by day 6 than they had been on day 3.

Yet another mechanism that we had to consider was the possibility raised by several studies that differences in homing could affect the ability of adoptively transferred lymphocytes to function properly in vivo (16, 42, 43). CD44 in particular has been reported to determine whether adoptively transferred clones were capable of preventing malaria as a result of infection with *Plasmodium yoelii* (44). The CTL lines and clones in our experiments do not show differences in the expression of CD44 that correlated with their avidity (data not shown). In addition, the enhanced ability of high-avidity CTL to reduce viral titers following adoptive transfer into infected SCID mice has been observed in three independently generated sets of lines as well as with CTL clones developed from those lines. To formally address the question of differences in the homing of high- and low-avidity CTL, however, CTL were labeled with fluorescent markers and transferred into SCID mice simultaneously infected with recombinant vaccinia. Ovaries harvested at 24 and 48 h showed no difference in the presence of labeled high- or low-avidity CTL in the ovary (Fig. 7), and, thus, no difference in the ability of either high- or low-avidity CTL to home to the site of infection, a result consistent with that seen in another model system (45).

In conclusion, two complementary mechanisms acting together appear to account for the greater efficacy of high-avidity CTL. First, high-avidity CTL recognize lower Ag densities present earlier in the course of infection of each cell, and, second, they initiate a more rapid lysis than low-avidity CTL. Both mechanisms allow them to eliminate cells infected with virus earlier. Early recognition and elimination of infected cells prevents the accumulation of virus and limits the extent of the resulting infection.

Acknowledgments

We thank Dr. Adrian Senderowicz for assistance in histological preparations and Drs. William E. Biddison and Jack Bennink for critical review of this manuscript and helpful comments.

References

1. Riddell, S. R., K. S. Watanabe, J. M. Goodrich, C. R. Li, M. E. Agha, and P. D. Greenberg. 1992. Restoration of viral immunity in immunodeficient humans by the adoptive transfer of T cell clones. *Science 257:238.*
2. Riddell, S. R., P. D. Greenberg, R. W. Overell, T. P. Loughran, M. J. Gilbert, S. D. Lupton, J. Agosti, S. Scheeler, R. W. Coombs, and L. Corey. 1992. Phase I study of cellular adoptive immunotherapy using genetically modified $CD8^+$ HIV-specific T cells for HIV seropositive patients undergoing allogeneic bone marrow transplant. *Hum. Gene Ther. 3:319.*
3. Holtappels, R., D. Thomas, J. Podlech, G. Geginat, H. P. Steffens, and M. J. Reddehase. 2000. The putative natural killer decoy early gene m04 (gp34) of murine cytomegalovirus encodes an antigenic peptide recognized by protective antiviral. *J. Virol. 74:1871.*
4. Kuzushima, K., M. Yamamoto, H. Kimura, Y. Ando, T. Kudo, I. Tsuge, and T. Morishima. 1996. Establishment of anti-Epstein-Barr virus (EBV) cellular immunity by adoptive transfer of virus-specific cytotoxic T lymphocytes from an HLA-matched sibling to a patient with severe chronic active EBV infection. *Clin. Exp. Immunol. 103:192.*
5. Roskrow, M. A., N. Suzuki, Y. Gan, J. W. Sixbey, C. Y. Ng, S. Kimbrough, M. Hudson, M. K. Brenner, H. E. Heslop, and C. M. Rooney. 1998. Epstein-Barr virus (EBV)-specific cytotoxic T lymphocytes for the treatment of patients with EBV-positive relapsed Hodgkin's disease. *Blood 91:2925.*
6. Khanna, R., S. Bell, M. Sherritt, A. Galbraith, S. R. Burrows, L. Rafter, B. Clarke, R. Slaughter, M. C. Falk, J. Douglass, et al. 1999. Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease. *Proc. Natl. Acad. Sci. USA 96:10391.*
7. Moss, D. J., R. Khanna, M. Sherritt, S. L. Elliott, and S. R. Burrows. 1999. Developing immunotherapeutic strategies for the control of Epstein-Barr virus-associated malignancies. *J. Acquired Immune Defic. Syndr. 21:80.*
8. Ehl, S., P. Klenerman, R. M. Zinkernagel, and G. Bocharov. 1998. The impact of variation in the number of $CD8^+$ T-cell precursors on the outcome of virus infection. *Cell. Immunol. 189:67.*
9. Lieberman, J., P. R. Skolnik, G. R. Parkerson, J. A. Fabry, D. M. Fong, B. Landry, and J. Kagan. 1994. Immunotherapy with ex vivo-expanded HIV-specific cytotoxic T lymphocytes in patients with CD4 counts of 100–400/mm^3. *AIDS Res. Hum. Retroviruses 10:S110.*
10. Tan, R., X. Xu, G. S. Ogg, P. Hansasuta, T. Dong, T. Rostron, G. Luzzi, C. P. Conlon, G. R. Screaton, A. J. McMichael, and S. Rowland-Jones. 1999. Rapid death of adoptively transferred T cells in acquired immunodeficiency immunodeficiency syndrome. *Blood 93:1506.*
11. Brodie, S. J., D. A. Lewinsohn, B. K. Patterson, D. Jiyamapa, J. Krieger, L. Corey, P. D. Greenberg, and S. R. Riddell. 1999. In vivo migration and function of transferred HIV-1-specific cytotoxic T cells. *Nat. Med. 5:34.*
12. Rosenberg, S. A., J. R. Yannelli, J. C. Yang, S. L. Topalian, D. J. Schwartzentruber, J. S. Weber, D. R. Parkinson, C. A. Seipp, J. H. Einhorn, and D. E. White. 1994. Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J. Natl. Cancer Inst. 86:1159.*
13. Schwartzentruber, D. J., S. S. Hom, R. Dadmarz, D. E. White, J. R. Yannelli, S. M. Steinberg, S. A. Rosenberg, and S. L. Topalian. 1994. In vitro predictors of therapeutic response in melanoma patients receiving tumor-infiltrating lymphocytes and interleukin-2. *J. Clin. Oncol. 12:1475.*
14. Merchant, R. E., N. G. Baldwin, C. D. Rice, and H. D. Bear. 1997. Adoptive immunotherapy of malignant glioma using tumor-sensitized T lymphocytes. *Neurol. Res. 19:145.*
15. Enomoto, A., K. Kato, H. Yagita, and K. Okumura. 1997. Adoptive transfer of cytotoxic T lymphocytes induced by CD86-transfected tumor cells suppresses

multi-organ metastases of C1300 neuroblastoma in mice. *Cancer Immunol. Immunother. 44:204.*
16. Ichii, S., Y. Imai, and T. Irimura. 1997. Tumor site-selective localization of an adoptively transferred T cell line expressing a macrophage lectin. *J. Leukocyte Biol. 62:761.*
17. Kim, S. J., M. Sadelain, J. S. Lee, R. H. Seong, Y. S. Yun, Y. J. Jang, and H. Y. Chung. 1999. Adoptive-transfer therapy of tumors with the tumor-specific primary cytotoxic T cells induced in vitro with the B7.1-transduced MCA205 cell line. *Cancer Immunol. Immunother. 47:257.*
18. Valmori, D., M. J. Pittet, D. Rimoldi, D. Lienard, R. Dunbar, V. Cerundolo, F. Lejeune, J. C. Cerottini, and P. Romero. 1999. An antigen-targeted approach to adoptive transfer therapy of cancer. *Cancer Res. 59:2167.*
19. Mackensen, A., S. Wittnebel, H. Veelken, C. Noppen, G. C. Spagnoli, and A. Lindermann. 1999. Induction and large-scale expansion of $CD8^+$ tumor specific cytotoxic T lymphocytes from peripheral blood lymphocytes by in vitro stimulation with CD80-transfected autologous melanoma cells. *Eur. Cytokine Network 10:329.*
20. Cardoso, A. A., J. P. Veiga, P. Ghia, H. M. Afonso, W. N. Haining, S. E. Sallan, and L. M. Nadler. 1999. Adoptive T-cell therapy for B-cell acute lymphoblastic leukemia: preclinical studies. *Blood 94:3531.*
21. Soda, H., K. Koda, J. Yasutomi, K. Oda, N. Takiguchi, N. Saito, and N. Nakajima. 1999. Adoptive immunotherapy for advanced cancer patients using in vitro activated cytotoxic T lymphocytes. *J. Surg. Oncol. 72:211.*
22. Alexander-Miller, M. A., G. R. Leggatt, and J. A. Berzofsky. 1996. Selective expansion of high or low avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy. *Proc. Natl. Acad. Sci. USA 93:4102.*
23. Gallimore, A., T. Dumrese, H. Hengartner, R. M. Zinkernagel, and H. G. Rammensee. 1998. Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides. *J. Exp. Med. 187:1647.*
24. Yee, C., P. A. Savage, P. P. Lee, M. M. Davis, and P. D. Greenberg. 1999. Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers. *J. Immunol. 162:2227.*
25. Zeh, I. I. I., H. J., D. Perry-Lalley, M. E. Dudley, S. A. Rosenberg, and J. C. Yang. 1999. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. *J. Immunol. 162:989.*
26. Takahashi, H., J. Cohen, A. Hosmalin, K. B. Cease, R. Houghten, J. Cornette, C. DeLisi, B. Moss, R. N. Germain, and J. A. Berzofsky. 1988. An immunodominant epitope of the HIV gp160 envelope glycoprotein recognized by class I MHC molecule-restricted murine cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA 85:3105.*
27. Matsui, S., J. D. Ahlers, A. O. Vortmeyer, M. Terabe, T. Tsukui, D. P. Carbone, L. A. Liotta, and J. Berzofsky. 1999. A model for $CD8^+$ CTL tumor immunosurveillance and regulation of tumor escape by CD4 T cells through an effect on quality of CTL. *J. Immunol. 163:184.*
28. Takeshita, T., H. Takahashi, S. Kozlowski, J. D. Ahlers, C. D. Pendleton, R. L. Moore, Y. Nakagawa, K. Yokomuro, B. S. Fox, D. H. Margulies, and J. A. Berzofsky. 1995. Molecular analysis of the same HIV peptide functionally binding to both a class I and a class II MHC molecule. *J. Immunol. 154:1973.*
29. Earl, P. L., A. W. Hugin, and B. Moss. 1990. Removal of cryptic poxvirus transcription termination signals from the human immunodeficiency virus type 1 envelope gene enhances expression and immunogenicity of a recombinant vaccinia virus. *J. Virol. 64:2448.*
30. Chakrabarti, S., M. Robert-Guroff, F. Wong-Staal, R. C. Gallo, and B. Moss. 1986. Expression of the HTLV-III envelope gene by a recombinant vaccinia virus. *Nature 320:535.*
31. Buller, R. M. L., and G. D. Wallace. 1985. Reexamination of the efficacy of vaccination against mousepox. *Lab. Anim. Sci. 35:473.*
32. Alexander, M. A., C. A. Damico, K. M. Wieties, T. H. Hansen, and J. M. Connolly. 1991. Correlation between CD8 dependency and determinant density using peptide-induced, Ld-restricted cytotoxic T lymphocytes. *J. Exp. Med. 173:849.*
33. Guidotti, L. G., and F. V. Chisari. 2000. Cytokine-mediated control of viral infections. *Virology 273:221.*
34. Valitutti, S., S. Muller, M. Dessing, and A. Lanzavecchia. 1996. Different responses are elicited in cytotoxic T lymphocytes by different levels of T cell receptor occupancy. *J. Exp. Med. 183:1917.*
35. Itoh, Y., and R. N. Germain. 1997. Single cell analysis reveals regulated hierarchical T cell antigen receptor signaling thresholds and intraclonal heterogeneity for individual cytokine responses of $CD4^+$ T cells. *J. Exp. Med. 186:757.*
36. Jackson, D. C., G. L. Ada, and R. Tha hla. 1976. Cytotoxic T cells recognize very early, minor changes in ectromelia virus-infected target cells. *Aust. J. Exp. Biol. Med. Sci. 54:349.*
37. Zinkernagel, R. M., and A. Althage. 1977. Antiviral protection by virus-immune cytotoxic T cells: infected target cells are lysed before infectious virus progeny is assembled. *J. Exp. Med. 145:644.*
38. Ehl, S., P. Klenerman, P. Aichele, H. Hengartner, and R. M. Zinkernagel. 1997. A functional and kinetic comparison of antiviral effector and memory cytotoxic T lymphocyte populations in vivo and in vitro. *Eur. J. Immunol. 27:3404.*
39. Brower, R. C., R. England, T. Takeshita, S. Kozlowski, D. H. Margulies, J. A. Berzofsky, and C. DeLisi. 1994. Minimal requirements for peptide mediated activation of $CD8^+$ CTL. *Mol. Immunol. 31:1285.*
40. Valitutti, S., S. Müller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature 375:148.*
41. Valitutti, S., and A. Lanzavecchia. 1997. Serial triggering of TCRs: a basis for the sensitivity and specificity of antigen recognition. *Immunol. Today 18:299.*
42. Brodie, S. J., D. A. Lewinsohn, B. K. Patterson, D. Jiyamapa, J. Krieger, L. Corey, P. D. Greenberg, and S. R. Riddell. 1999. In vivo migration and function of transferred HIV-1-specific cytotoxic T cells. *Nat. Med. 5:34.*
43. Santi, A. D., P. L. Hermonat, A. Ravaggi, S. Bellone, C. Cowan, S. Korourian, S. Pecorelli, M. J. Cannon, and G. P. Parham. 2000. Development, characterization and distribution of adoptively transferred peripheral blood lymphocytes primed by human papillomavirus 18 E7-pulsed autologous dendritic cells in a patient with metastatic adenocarcinoma of the uterine cervix. *Eur. J. Gynaecol. Oncol. 21:17.*
44. Rodrigues, M., R. S. Nussenzweig, P. Romero, and F. Zavala. 1992. The in vivo cytotoxic activity of $CD8^+$ T cell clones correlates with their levels of expression of adhesion molecules. *J. Exp. Med. 175:895.*
45. Nugent, C. T., D. J. Morgan, J. A. Biggs, A. Ko, I. M. Pilip, E. G. Pamer, and L. A. Sherman. 2000. Characterization of $CD8^+$ T lymphocytes that persist after peripheral tolerance to a self antigen expressed in the pancreas. *J. Immunol. 164:191.*