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Immunology

# Selective expansion of high- or low-avidity cytotoxic T lymphocytes and efficacy for adoptive immunotherapy

**(viral immunity/virus clearance/T-cell therapy/determinant density/antigen dose)**

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**ABSTRACT** **The conventional approach to cytotoxic T-lymphocyte (CTL) induction uses maximal antigen concentration with the intent of eliciting more CTL. However, the efficacy of this approach has not been systematically explored with regard to the quality of the CTLs elicited or their *in vivo* functionality. Here, we show that a diametrically opposite approach elicits CTLs that are much more effective at clearing virus. CTLs specific for a defined peptide epitope were selectively expanded with various concentrations of peptide antigen. CTLs generated with exceedingly low-dose peptide lysed targets sensitized with >100-fold less peptide than CTLs generated with high-dose peptide. Differences in expression of T-cell antigen receptors or a number of other accessory molecules did not account for the functional differences. Further, high-avidity CTLs adoptively transferred into severe combined immunodeficient mice were 100- to 1000-fold more effective at viral clearance than the low-avidity CTLs, despite the fact that all CTL lines lysed virus-infected targets *in vitro*. Thus, the quality of CTLs is as important as the quantity of CTLs for adoptive immunotherapy, and the ability to kill virally infected targets *in vitro* is not predictive of *in vivo* efficacy, whereas the determinant density requirement described here is predictive. Application of these principles may be critical in developing effective adoptive cellular immunotherapy for viral infections and cancer.**

The approach to elicit and expand cytotoxic T lymphocytes (CTLs) by most investigators to date has been based on the idea that increased peptide would yield stronger CTL responses. In such cases, a stronger response is likely attributable to activation of a larger number of peptide-specific precursors. Clever methods, such as the use of transporter-mutant cells that can be coated homogeneously with a desired peptide (1), have been developed to achieve high determinant density to elicit more CTLs. The question remains, however, of whether elicitation and expansion of every potential precursor are the most effective mechanisms for generating an optimal CTL response. In other words, is the quality of the responding CTLs as, or perhaps even more, important for *in vivo* function than the quantity of CTLs present? Several laboratories are currently investigating adoptive transfer of *in vitro* expanded autologous CTLs for treatment of a number of disease states, including cytomegalovirus (2, 3), human immunodeficiency virus type 1 (HIV-1) (4), and cancer (5, 6). Thus, the question posed above becomes of utmost importance, but it has not been investigated previously. Consequently, we were interested in determining the functional attributes that would confer *in vivo* efficacy to CTLs; in particular, we wanted to discover the relationship among stimulating antigen dose, *in vitro* phenotype, and *in vivo* function. Here, we show that CTL avidity is critical for *in vivo* efficacy and that the best approach for eliciting such CTLs is diametrically opposed to the currently accepted paradigm and requires exceedingly low concentrations of peptide. A panel of CTL lines, generated by stimulation with various concentrations of I10 peptide, the immunodominant epitope from HIV-1 IIIB gp160 (7), exhibited significant differences in their determinant density requirements, with low-dose stimulation resulting in >100-fold greater sensitivity to peptide. Adoptive transfer of these lines into severe combined immunodeficient (SCID) mice innoculated with a recombinant vaccinia virus expressing the gp160 protein demonstrated that viral clearance correlated with the determinant density requirement of the lines, despite the fact that both types of CTLs effectively killed virus-infected cells *in vitro*. Thus, the ability of CTLs to clear virus *in vivo* cannot be predicted simply by the ability of CTLs to kill infected targets. The results demonstrate that the concentration of peptide used to expand CTLs for adoptive immunotherapy will significantly affect CTLs *in vivo* efficacy and that by regulating the concentration of antigen *in vitro*, one can selectively generate CTLs with discrete functional avidities and distinct *in vivo* activity in viral clearance.

## MATERIALS AND METHODS

**Mice, Antibodies, and Cell Lines.** BALB/c mice were purchased from The Jackson Laboratory and SCID mice (BALB/c background) were purchased from the Frederick Cancer Research and Development Center (Frederick, MD). Fluorescein isothiocyanate-conjugated anti-CD8$\alpha$ (clone 53–6.7), anti-LFA-1 (clone 2D7), anti-Thy-1.2 (clone 53–2.1), anti-CD3 (clone 145–2C11), anti-TCR$\alpha\beta$ (clone H57–597), and anti-CD49 (clone R1–2) monoclonal and goat anti-mouse immunoglobulin polyclonal antibodies were purchased from PharMingen. F23.1, specific for $V_\beta 8$, was a kind gift of Richard Hodes (National Institute on Aging, Bethesda). 15-12, derived by transfection of BALB/c 3T3 cells, expresses the gp160 protein from the IIIB strain of HIV-1 (7). P815 is a DBA/2-derived mastocytoma.

**Peptides.** Peptides were synthesized on an automated peptide synthesizer (model no. 430A, Applied Biosystems) using t-boc chemistry (8) and were purified as described (9).

**Recombinant Vaccinia Virus.** vPE-16 is a recombinant vaccinia virus that expresses the gp160 protein from the IIIB strain of HIV-1 (10) and was a kind gift of Patricia Earl and Bernard Moss (National Institute of Allergy and Infectious Diseases, Bethesda). WR is a control vaccinia construct that does not express gp160. For infection of target cells, vaccinia was added to $1 \times 10^6$ P815 cells at a multiplicity of infection of 50 in 1 ml of cell culture medium 16 hr before assay and incubated at 37°C.

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Abbreviations: HIV-1, human immunodeficiency virus type 1; CTL, cytotoxic T lymphocyte; SCID, severe combined immunodeficient; MHC, major histocompatibility complex; TCR, T-cell antigen receptor.

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***In Vivo* Reconstitution and Vaccinia Clearance in SCID Mice.** H-2$^d$ SCID mice were inoculated i.v. via the tail vein with $1 \times 10^7$ CTLs in 100 μl of phosphate-buffered saline (PBS). CTLs were used for reconstitution on day 4 after stimulation. Immediately after CTL administration, $5 \times 10^7$ plaque-forming units of recombinant vaccinia was delivered in 200 μl of PBS i.p. Three days later, mice were killed and tissues were harvested. Samples were frozen at −70°C until analysis as described by Buller and Wallace (11).

**Generation of CTL Lines.** Responding BALB/c spleen cells ($7.5 \times 10^6$) from mice previously immunized with a recombinant vaccinia expressing the gp160 protein from the HIV-1 IIIB strain were co-cultured either with $3.5 \times 10^6$ stimulating BALB/c splenocytes [3000 rads (1 rad = 0.01 Gy)] pulsed with various concentrations (100, 0.1, or 0.0001 μM) of I10 peptide or in the presence of 1 μM free peptide in a 24-well plate containing 2 ml of a 1:1 mixture of RPMI 1640 medium and Eagle–Hanks' amino acid (EHAA) medium supplemented with L-glutamine, sodium pyruvate, nonessential amino acids, penicillin, streptomycin, $5 \times 10^{-5}$ M 2-mercaptoethanol, 10% fetal calf serum, and 10% T-stim (Collaborative Biomedical Products, Bedford, MA). CTL lines were established from primary cultures and were maintained 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 pulsed with the appropriate concentration of I10 peptide.

**$^{51}$Cr Release Assay.** The $^{51}$Cr release assay was carried out as described (12). Target cells ($1 \times 10^6$) were labeled with 300 μCi (1 Ci = 37 GBq) of $Na_2\,^{51}CrO_4$ in 200–250 μl for 2 hr at 37°C. Where appropriate, targets were pulsed with peptide during labeling. Cells were then washed and added to wells along with the appropriate number of effector cells in 96-well round-bottom plates. After 4 hr, supernatants were harvested and counted in an ISOMEDIC gamma counter (ICN). The mean of triplicate samples and percent of $^{51}$Cr release was calculated.

**Flow Cytometry.** For flow cytometric analysis, $2 \times 10^5$ cells were washed and resuspended in PBS containing 0.2% bovine serum albumin and 0.1% sodium azide. Cells were incubated on ice with the appropriate antibody for 30 min and washed. Where necessary, a secondary reagent was then added for an additional 30 min, and the cells were again washed. Samples were analyzed on a FACScan (Becton Dickinson). Background staining was ascertained with an irrelevant antibody.

## RESULTS

**CTL Lines Generated via Stimulation with I10-Pulsed Splenocytes Have Determinant Density Requirements That Correlate with the Stimulating Concentration of Peptide.** To stringently address the effect of stimulating antigen dose on the functional properties of responding CTLs, a panel of lines was generated by *in vitro* restimulation of splenocytes from BALB/c mice immunized with $1 \times 10^7$ plaque-forming units of vPE-16, which is a recombinant vaccinia construct that expresses the gp160 envelope protein from HIV-1 IIIB (10). The immunodominant epitope recognized by CTLs generated in this manner is I10 (RGPGRAFVTI) (7, 9). Splenocytes pooled from two immunized mice were divided and restimulated with autologous splenocytes pulsed with various concentrations of I10 peptide (100, 0.1, or 0.0001 μM) or with soluble I10 peptide (1 μM) added directly to the stimulation culture. We hypothesized that the inclusion of 1 μM free peptide during the stimulation may allow for maximal loading of H-2D$^d$ molecules compared with cells receiving a 2- to 3-hr pulse. However, the possibility that the phenotype of lines stimulated with soluble peptide is at least in part due to the presentation of I10 by the CTL to either itself or other CTLs cannot be ruled out. No CTL lines could be generated in the absence of peptide. The density of peptide–major histocompatibility complex (MHC) complexes on the target cells was titrated by pulsing with graded concentrations of I10 peptide. The peptide–MHC determinant density requirements of lines generated correlated with the amount of antigen used for stimulation in that lines generated with respectively lower amounts of I10 lysed target cells pulsed with lower doses of I10 (Fig. 1*A*). Specifically, lines generated with 0.0001 μM-pulsed stimulators lysed targets pulsed with 100-fold lower concentrations of peptide than lines generated on 1 μM free peptide. The line generated on 100 μM-pulsed stimulators required >10-fold more peptide to lyse targets than the line generated on 0.0001 μM.

In a separate panel of CTL lines generated from vPE-16-immunized mice, responder cells were stimulated with either 15-12, a cell line derived by transfection of BALB/c 3T3 cells with gp160 (7), or with 1 μM soluble I10 peptide. In agreement with the previous finding, these lines vary in their determinant density requirements by about 100-fold (Fig. 1*B*). The line generated using 15-12 cells is more similar in its determinant density requirement to lines generated on low-dose peptide, suggesting that in this case, endogenous presentation results in relatively low determinant density presentation. This interpretation is in agreement with the low level of gp160 expression by 15-12 cells, detectable at the mRNA level but not at the protein level by Western blot analysis (J. Cohen and R. Germain, personal communication). The correlation between stimulating antigen dose and determinant density requirement was reproduced in two additional independent panels of lines generated by stimulation with graded concentrations of peptide (data not shown). Also, as expected for T cells of different avidity (12, 13), the sensitivity to inhibition by anti-CD8 antibody correlated with increasing determinant density requirements (data not shown). Together, the above data demonstrate that the avidity of the CTL population obtained

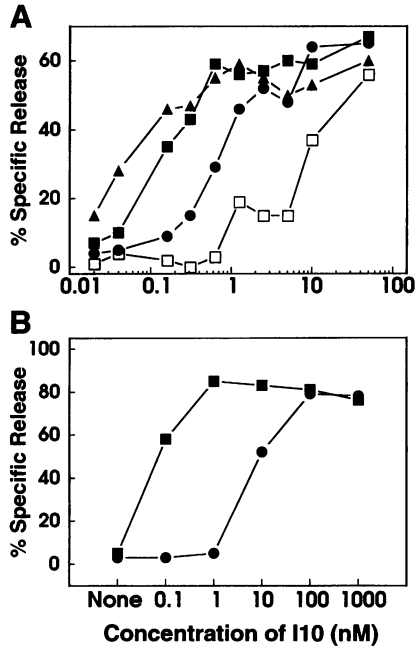


FIG. 1. The concentration of peptide antigen used for stimulation determines the determinant density requirement of the resulting line. (*A*) CTL lines were generated by restimulation of splenocytes from BALB/c mice immunized with vPE-16. Lines were generated with stimulators pulsed with 100 μM (●), 0.1 μM (■), or 0.0001 μM (▲) of I10 or by addition of 1 μM I10 directly into the cultures (□). After multiple stimulation cycles, CTL lines were assayed for lysis of P815 target cells pulsed with each of 10 different concentrations of I10 peptide at an effector-to-target cell ratio (E/T) of 10:1. (*B*) CTL lines were generated by restimulation of vPE-16-immune splenocytes with 1 μM soluble I10 peptide (●) or with 15-12 cells that express gp160 endogenously (■). CTLs were tested at an E/T of 10:1 with targets as in *A*.

during an immune response is a direct result of the antigen density presented by the stimulating antigen-presenting cell (APC). In this context, we have used avidity to mean sensitivity to low peptide–MHC complex determinant density on the target cells, with the understanding that other mechanisms may apply as well. Most of the following analyses were performed with additional lines, with one line shown as representative.

**Assessment of Cell Surface Molecules Expressed by CTLs with Differing Determinant Density Requirements.** As differences in the expression of T-cell antigen receptors (TCRs) or other accessory molecules could contribute to the observed differences in avidity, the CTL lines were assayed for expression of a number of cell surface molecules that have been implicated in CTL function, including CD8, LFA-1, Thy-1.2, and VLA-4 (Table 1). Although there are differences in the expression of some molecules (e.g., TCRs), they do not appear to correlate with functional phenotype, although we cannot exclude from the fluorescence-activated cell sorter analysis the formal possibility that a small subset of cells with skewed TCR expression contributes disproportionately to the response. These results suggest that other mechanisms, including variation in TCR affinities or differences in the number of TCRs that must be engaged for activation to occur, may account for the *in vitro* functional differences observed among the CTL lines.

A previous study (14) from our lab identified $V_\beta 8$ as a predominant $V_\beta$ chain employed by I10-specific CTLs, although other $V_\beta$ chains can be used. To determine whether the stimulating antigen dose influenced the TCR repertoire of the CTLs, the lines were analyzed for expression of $V_\beta 8$. Interestingly, the two higher avidity lines contained only a small percentage of $V_\beta 8^+$ CTLs (0.0001 $\mu$M line < 15% and 0.1 $\mu$M line < 4%), whereas the lines generated on 100 $\mu$M-pulsed stimulators or 1 $\mu$M soluble peptide contained 55% and >99% $V_\beta 8^+$ cells, respectively. This correlation of $V_\beta 8$ usage with lower-avidity cells was also observed in an independent panel of lines (data not shown). The difference in $V_\beta$ usage implies that different TCRs are used by the lines. Because the lines had undergone 20 rounds of stimulation, it is likely that all of the cells are antigen-specific despite different $V_\beta$ usage, consistent with the finding that TCRs with different $V_\beta$s can recognize the I10-$D^d$ complex (14).

**Divergence Among the Lines in the Number of Engaged TCR Required for Triggering May Contribute to the Determinant Density Requirement of the CTL Lines.** To determine whether differences in the number of TCRs that must be engaged for activation contributed to the observed variation in determinant density requirements, anti-CD3 antibody was used to direct lysis by the CTL lines in the absence of specific peptide antigen. Anti-CD3 has been shown to promote lysis through Fc receptor binding on the P815 target cell and concurrent binding to CD3 on the CTL (15). Because activation through CD3 bypasses the interaction of TCRs with antigen, differences in TCR affinity cannot influence the dose-response curve to anti-CD3 antibody. If indeed the differences in determinant density requirements are solely the result of variation in the TCR affinity, then the lysis obtained using anti-CD3 should be equivalent. However, Fig. 2 shows

Table 1. Expression of cell surface molecules on the CTL lines with various avidities

| Concentration of I10 used to generate line | Δ mean fluorescence | | | | |
|---|---|---|---|---|---|
| | TCR | CD8 | Thy-1.2 | LFA-1 | VLA-4 |
| 0.0001 $\mu$M | 102 | 160 | 846 | 1154 | 20 |
| 0.1 $\mu$M | 174 | 238 | 731 | 1354 | 14 |
| 100 $\mu$M | 101 | 204 | 941 | 1355 | 13 |
| 1 $\mu$M (soluble) | 71 | 172 | 794 | 1335 | 18 |

Δ mean fluorescence is calculated as the mean fluorescence obtained with the antibody minus the mean background fluorescence.

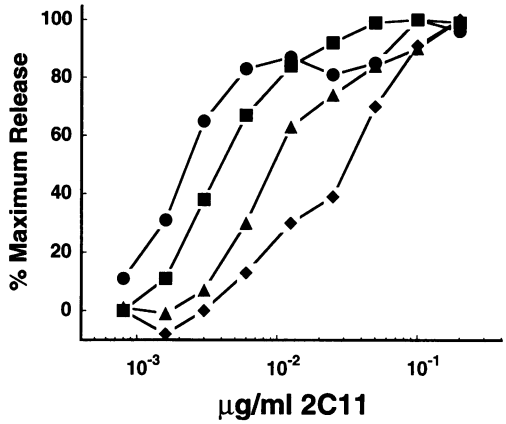


FIG. 2. CTL lines of different avidities respond with differing sensitivities to anti-CD3 antibody in a redirected lysis assay. CTL lines stimulated with 0.0001 $\mu$M-pulsed (●), 0.1 $\mu$M-pulsed (■), 100 $\mu$M-pulsed (▲), or 1 $\mu$M soluble I10 (◆) peptide were tested for recognition of P815 target cells incubated with anti-CD3 (2C11) antibody at titered concentrations at an effector-to-target cell ratio of 5:1. The presence of anti-CD3 antibody did not affect the spontaneous release of the target cells.

that the dose-response curves among the lines are unexpectedly distinct. Although there is variation in TCR expression among the lines (Table 1), this level of TCR expression does not explain the differences in the dose-response curves to anti-CD3—e.g., the levels of TCR expression for the lines generated with 100 $\mu$M peptide and 0.0001 $\mu$M peptide have significantly different dose-response curves yet have equivalent TCR expression, 101 vs. 102 mean fluorescence intensity, respectively. This finding suggests that the higher avidity lines require fewer TCRs to be engaged for activation to occur, possibly because of differences in coupling of TCRs to downstream signaling mechanisms. This factor likely contributes to the apparent differences in the concentration of antigen needed for target cell sensitization. However, whether the 8-fold difference (average of three independent assays) in the concentration of anti-CD3 required to achieve lysis detected between the lines of highest and lowest avidity can fully account for the 100-fold difference in determinant density required for lysis must be determined by cloning of the TCR genes and direct measurements of TCR affinity of representative CTL from each line.

**Recognition of Endogenously Expressing Targets by CTL Lines with Different Determinant Density Requirements.** In view of the reports that CTL lines generated by *in vitro* primary stimulation with high concentrations of peptide often fail to lyse targets endogenously expressing antigen (16, 17), the CTL lines were assessed for recognition of targets infected with recombinant vaccinia virus expressing gp160. All four lines recognized vPE-16-infected P815 target cells, although the maximal lysis achieved was greater with the higher affinity lines (Fig. 3). Recognition of vaccinia-infected targets demonstrated that all of the lines, regardless of avidity, could recognize endogenously processed and presented antigen. This observation was intriguing in light of the appreciable differences in the concentration of peptide required for target cell sensitization. To determine the *in vivo* implications of these *in vitro* findings, the CTL lines were tested for the ability to clear virus in mice.

***In Vivo* Clearance of Recombinant Vaccinia Expressing gp160 Correlates with the Determinant Density Requirements of the CTL Lines.** A primary function of CTLs *in vivo* is clearance of viral infections (18, 19). We hypothesized that even though both low- and high-avidity CTLs can kill targets infected with recombinant vaccinia, those CTLs that can recognize cells early in infection, before much virus replication

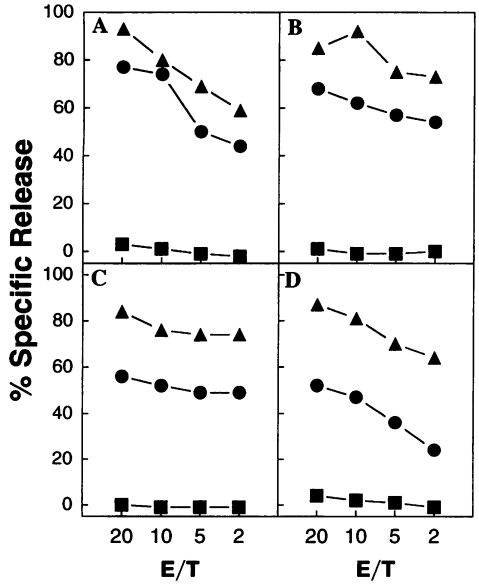


FIG. 3. High- and low-avidity CTL lines efficiently recognize targets expressing I10 endogenously. CTLs generated on 0.0001 μM I10-pulsed stimulators (*A*), 0.1 μM I10-pulsed stimulators (*B*), 100 μM I10-pulsed stimulators (*C*), and 1 μM soluble I10 (*D*) were tested on vPE-16-infected P815 cells (●) and control WR-infected cells in the absence (■) or presence (▲) of 10 μM I10 peptide. All four CTL lines recognized endogenously expressing targets.

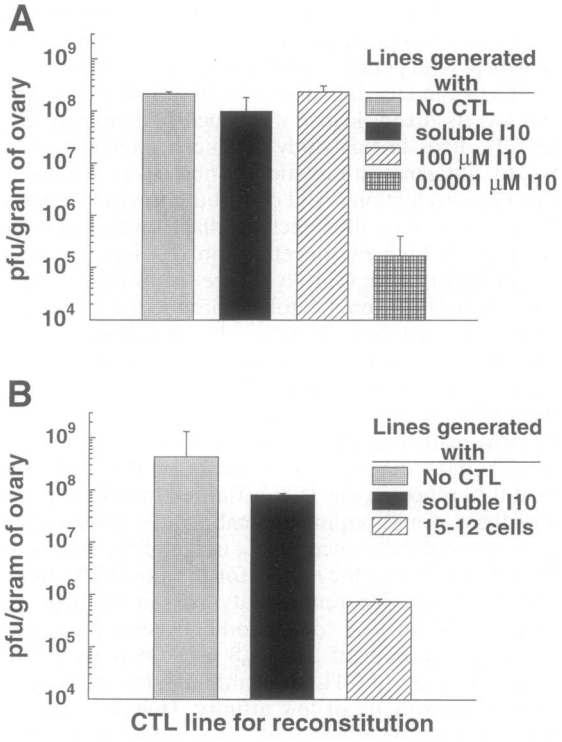


FIG. 4. High-avidity lines efficiently clear virus *in vivo*, whereas low-avidity lines are ineffective. CTLs ($1 \times 10^7$) were injected i.v. and $5 \times 10^7$ plaque-forming units of recombinant vaccinia expressing gp160 were injected i.p. into H-$2^d$ SCID mice. On day 3, mice were killed and ovaries were harvested, as the virus has a tropism for the ovaries. Vaccinia present in the tissues was determined by titering on BSC-1 cells. (*A*) Lines tested were generated by stimulation with 0.0001 μM- or 100 μM-pulsed splenocytes or in the presence of 1 μM soluble peptide. Results shown are the titers obtained in a representative experiment with three to five mice per group. Similar results were obtained in two additional independent experiments. *P* values calculated using Student's *t* test were as follows: mice receiving the line generated on 0.0001 μM I10-pulsed stimulators vs. mice receiving no CTLs, $P = 0.00002$ or mice receiving 100 μM-stimulated CTLs, $P = 0.00005$, or mice receiving CTLs generated on 1 μM soluble I10, $P = 0.004$. Results obtained from mice receiving low-avidity lines vs. no CTLs had *P* values of >0.05. (*B*) Lines used for reconstitution were generated by stimulation with 1 μM soluble peptide or with the 15-12 transfectant that expresses the peptide determinant endogenously. *P* values were as follows: mice receiving CTLs generated on 15-12 cells vs. mice receiving no CTLs, $P = 0.049$, or vs. mice receiving CTLs generated with 1 μM soluble I10, $P = 0.0019$. For mice receiving no CTLs versus CTLs generated with 1 μM soluble I10, $P = 0.35$.

has occurred, and therefore before high levels of viral peptide–MHC complexes are on the cell surface, might be more efficient at clearing virus. To test this hypothesis and to assess the *in vivo* relevance of these CTLs with differing functional avidities, the lines were compared for their ability to clear virus from SCID mice. Mice received either $1 \times 10^7$ CTLs or PBS alone as a mock control via tail vein injection. Concurrently, mice received $5 \times 10^7$ plaque-forming units of vPE-16 by i.p. injection. CTLs generated on 0.0001 μM-pulsed stimulators reduced the amount of virus in the ovary by 1000-fold more than CTLs generated on 1 μM soluble I10 or 100 μM-pulsed stimulators ($P = 0.004$ and 0.00005, respectively) (Fig. 4*A*). Similar results were obtained in two additional experiments using this panel of lines. In addition, an independent panel of lines generated by stimulation with either 1 μM soluble I10 or 15-12 cells and displaying disparate determinant density requirements (Fig. 1*B*) showed a similar difference for *in vivo* viral clearance (Fig. 4*B*) (for *P* values, see Fig. 4 legend). Thus, the correlation between determinant density requirement and *in vivo* efficacy has been observed in two independent panels of lines. Therefore, the dose of peptide used to stimulate CTLs will directly affect the ability of those CTLs to function *in vivo* to clear virus.

## DISCUSSION

In this study, CTLs were generated by repeated *in vitro* stimulation of immune cells with distinct antigen doses. CTLs generated in this manner display significant and reproducible differences in their antigen dose requirements for optimal target cell lysis. Although CTLs with a broad range of determinant density requirements have been observed following cloning of polyclonal responses, this study is the first to demonstrate a mechanism for the selective bulk expansion of CTL populations possessing a distinct *in vivo* viral clearance capacity and *in vitro* peptide-MHC requirement. A previous observation of increased lytic activity after stimulation with low-dose antigen is likely explained by the data presented here (20). The functional phenotypes of the CTL lines as measured by their determinant density requirements appear to be quite stable and do not change. The *in vitro* differences in determinant density requirements of the lines in our study correlate directly with the lines' abilities to clear virus *in vivo*. CTLs requiring high determinant density for target cell recognition were ineffective in reducing viral burden in SCID mice inoculated with recombinant vaccinia virus expressing HIV-1 gp160, even though both lines lysed virus-infected targets *in vitro*. In contrast, CTLs capable of lysing targets bearing low antigen densities could reduce viral titers by 100- to 1000-fold, compared with lower-avidity CTLs.

The data obtained with the CTL lines of differing avidity demonstrate that *in vitro* recognition of targets infected with virus is not necessarily reflective of the ability of those same CTLs to clear viral infection *in vivo*. This is an important finding, as recognition of endogenously expressed antigen has been regarded as an indicator of *in vivo* relevance (21). Based on the data in our study, we propose that measurement of determinant density requirement may be a better predictor of *in vivo* efficacy for adoptive immunotherapy. We conclude that the quality of CTLs is as important as the quantity for clearing

viral infection. This result is consistent with the hypothesis that CTLs 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 CTLs that can kill only targets already loaded with much viral progeny (22, 23).

The CTL lines in this study produced interferon-$\gamma$ in response to antigenic stimulation. Thus, it is possible that interferon-$\gamma$ production could contribute to viral clearance in addition to lysis of virally infected cells. However, the thresholds of peptide antigen concentration required for interferon-$\gamma$ production and CTL lysis are equivalent (data not shown). Therefore, even if cytokine production contributes to the observed clearance *in vivo*, the threshold of antigen concentration required for its production is still determined by CTL avidity and is selected for by *in vitro* stimulation with different antigen concentrations.

The most ready explanation for the observed differences in the determinant density requirements of the CTL lines is inherent differences in the TCR affinities, but direct measurement of these would require recombinant soluble TCR molecules. Observed differences in $V_\beta$ usage of the lines support this hypothesis in that they allow for the possibility that cells with TCRs of very different affinity exist in the high- versus low-avidity CTL lines. A correlation between a high determinant density requirement and $V_\beta 8$ usage was found in two independent panels of CTL lines, although not all TCRs using $V_\beta 8$ will necessarily be of low affinity. However, alternative mechanisms must be considered. Two additional possibilities were differences in the level of expression of the TCR itself or in various accessory molecules. No differences in the expression of CD8, LFA-1, VLA-4, or Thy-1.2 were found. Although there are differences in the level of TCRs expressed, the pattern of expression does not correlate with the functional phenotype of the lines (Table 1 and Fig. 1). In a previous report, differences in the level of CD44 expression were shown to affect *in vivo* clearance of adoptively transferred CTL clones (24). Relative CD44 expression among the lines in the current study (tested in two independent panels of lines) varied over time in culture (data not shown), but *in vivo* clearance never correlated with such differences. Thus, differences in homing to targets are unlikely to account for the results. Third, the trivial explanation of a difference in MHC restriction was ruled out because both high- and low-avidity lines are restricted by H-2D$^d$ (data not shown). A fourth alternative mechanism is suggested by Kwan-Lim *et al.* (25), who reported differences among a panel of hybridomas in their response to plate-bound anti-CD3 antibody, suggesting a difference in the number of engaged TCRs required for activation. Redirected lysis experiments showed that differences in the number of TCRs that must be engaged for activation do appear to contribute, at least in part, to the differences in determinant density requirements of these lines, as the dose-response curves to anti-CD3 antibody were modestly shifted among the lines (Fig. 2). This surprising result suggests that differences may be present in the signaling threshold required to achieve activation of the various lines or in the efficiency of coupling of the TCRs to downstream signaling events. Therefore, the sensitivity to determinant density of a CTL is likely a combination of several factors, including TCR affinity and the threshold level of TCR engagement required for signaling.

Responding cells used for generation of the lines in this study were initially activated *in vivo* by immunization with a recombinant vaccinia expressing gp160. Thus, even lower avidity cells can be activated during an endogenous infection. As previous attempts to generate primary responses by *in vitro* activation of naive splenocytes have been unsuccessful, it is unlikely that these CTLs were activated *in vitro* from naive precursors (data not shown). Elicitation of these CTLs *in vivo* does not necessitate that they are effective for viral clearance, as seems to be the case for the CTLs studied here.

In addition to obvious practical research applications (e.g., generation of lines for *in vitro* study), these findings have important implications for clinical therapy. A number of groups have undertaken clinical trials in which peripheral blood mononuclear cells, presumably containing CTLs specific for the antigen of interest, are removed from the patient, expanded *in vitro*, and reinfused. This procedure is being used for HIV positive patients (4), immunosuppressed patients combating cytomegalovirus (2), and patients with certain cancers (5, 6, 26). The present study suggests that the manner in which these CTLs are stimulated *in vitro* will have profound effects on their efficacy *in vivo*. Selective expansion of bulk CTLs using stimulators expressing relatively low determinant density as demonstrated in the current study, as opposed to cloning, may be optimal because large numbers of CTLs can be obtained quickly and lines are polyclonal. The latter attribute should be beneficial as adoptive immunotherapy using a monoclonal CTL population can readily allow for viral escape (27). Conversely, elicitation of CTLs that require high determinant density may be beneficial for selective killing of tumor cells in situations in which the antigen of interest is a self-peptide that is overexpressed in the target cell, as is the case in melanoma (28). CTLs generated on high-dose antigen that may differentially recognize a tumor cell with supranormal expression while sparing wild-type cells would be ideal in this situation. It is important to note that high and low determinant density are relative and will vary for different peptides, depending on their affinity for MHC and the repertoire of TCRs available from which to select. Thus, optimal peptide concentrations must be determined empirically. In conclusion, the data presented here provide evidence that the quality is as important as the quantity of CTLs and that CTLs with discrete avidities can be preferentially selected and expanded in accordance with their desired therapeutic usage *in vivo*. This ability should be a powerful tool in the use of CTLs for adoptive therapy of disease.

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