The Journal of Immunology

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J Immunol 1999; 162:989-994; ;
http://www.jimmunol.org/content/162/2/989

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

High Avidity CTLs for Two Self-Antigens Demonstrate Superior In Vitro and In Vivo Antitumor Efficacy

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A majority of the human tumor-associated Ags characterized to date are derived from nonmutated "self"-proteins. Little is currently understood about the nature of the self-reactive lymphocytes that recognize these Ags. We recently characterized two nonmutated tumor-associated Ags for the B16 murine melanoma: tyrosinase-related protein-2 (TRP-2) and the endogenous retroviral envelope protein, p15E. We previously reported that both TRP-2 and p15E reactive CTL could be detected in the spleens of naive animals after a single in vitro stimulation using 10^{-5}–10^{-6} M of the appropriate K^b-binding 9-amino acid epitope. In this report we show that the CTL found in naive animals are low avidity lymphocytes, that respond only to high concentrations of peptide in vitro. We demonstrate that titration of in vitro-stimulating peptide to limiting concentrations distinguishes qualitative differences in the lymphocyte reactivity to these two Ags between vaccinated and unvaccinated animals. We further demonstrate that in vitro expansion of CTL in either high or low concentrations of stimulating peptide generated CTL cultures with different avidities for the relevant epitopes. CTL expanded in low concentrations demonstrated higher avidity for peptide-pulsed targets and better tumor recognition, when compared to CTL generated in the presence of high concentrations of Ag. More importantly, high avidity CTL demonstrated superior in vivo antitumor activity. These results demonstrate that qualitative differences in the CTL that recognize these two self-Ags are critically important to their in vitro and in vivo anti-tumor efficacy. ***The Journal of Immunology,*** **1999, 162: 989–994.**

One of the most significant advances in the field of tumor immunology has been the identification and characterization of the tumor-associated Ags that are recognized by T cells (1). Several of the melanoma-associated Ags described to date are derived from normal nonmutated melanocyte lineage differentiation Ags (2, 3). This class of tumor-associated Ags, including MART-1, gp100, tyrosine-related protein-1, and tyrosine-related protein-2, are shared Ags, recognized in a MHC-I-restricted fashion by lymphocytes from many different patients. The shared nature of these Ags has made them attractive candidates for clinical immunotherapeutic strategies for patients with cancer. Recent trials evaluating vaccination against these shared Ags in combination with systemic IL-2 have demonstrated promising results (4). Despite these early advances, relatively little is understood about the character of the autoreactive T cells that recognize these nonmutated self Ags. Until recently, few accurate murine models of shared, nonmutated tumor-associated Ags existed (5). We recently characterized two tumor-associated Ags for the B16 murine melanoma: tyrosinase-related protein-2 (TRP-2)[2] and the endogenous retroviral envelope protein p15E and found that TRP-2 and p15E-reactive CTL could be detected in the spleens of naïve animals after a single in vitro stimulation using 10^{-5}–10^{-6} M of the appropriate 9-amino acid peptide epitope (6, unpublished data). In this report, we further characterize the autoreactive cytotoxic T lymphocytes for these two Ags. Specifically, we show that the CTL found in naive animals are low avidity CTL, which respond only to high concentrations of minimal determinant peptide. We further show that titration of the in vitro-stimulating peptide concentration can demonstrate differences in the Ag reactivity between vaccinated and unvaccinated animals. We also show that selective expansion of either high or low avidity CTL is possible by controlling the concentration of in vitro-stimulating Ag and that these differences in CTL avidity correlate with the in vivo antitumor efficacy in an adoptive transfer model.

Materials and Methods

Animals

Female C57BL/6 mice were obtained from the Frederick Cancer Research and Development Center, National Institutes of Health (Frederick, MD) or Charles River Laboratories (Raleigh, NC) at 4–6 wk of age, housed in a facility with surveillance murine Ab production (MAP) testing and pathogen screening, fed ad libitum, and utilized at 8–12 wk of age.

Cell lines and peptides

The murine melanoma B16 is a spontaneously arising melanoma of C57BL/6 mice propagated by Dr. I. J. Fidler (M. D. Anderson Cancer Center, Houston, TX). B16 GM-CSF tumor cell line was a kind gift of Dr. Drew Pardoll (Johns Hopkins University, Baltimore, MD). The MCA fibrosarcomas, MCA 101, 102, and 205, were generated in our laboratory by the injections of 0.1 ml of 1% 3-methylcholanthrene in sesame oil. The MC-38 colon adenocarcinoma was generated by administration of oral 1,2-dimethhydrazine in C57BL/6 mice. All tumor cells lines were initially maintained by in vivo transplantation of early passage tumor, and subsequently by bulk culture in complete medium (RPMI 1640, 10% FCS, 0.5 μg/ml fungizone, 50 μg/ml gentamicin (Biofluids, Rockville, MD), 100 U/ml penicillin, 0.03% glutamine, 0.1 mM nonessential amino acids, 100 μg/ml streptomycin, 55 μM 2-ME (Life Technologies, Grand Island, NY), and 1 mM sodium pyruvate (Biofluids). 293K^b cells are a transformed human renal epithelial line stably transfected to express the murine

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Received for publication July 20, 1998. Accepted for publication October 7, 1998.

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[2] Abbreviations used in this paper: TRP-2, tyrosine-related protein-2; GM-CSF, granulocyte-macrophage colony-stimulating factor; pCTL, precursor cytotoxic T lymphocytes.

0022-1767/99/$02.00

class I molecule K^b. TRP-$2_{180-188}$ (SVYDFFVWL) and p15$E_{604-611}$ (KSPWFTTL) peptides were synthesized and purified by HPLC to greater than 95% purity by Peptide Technologies (Rockville, MD).

Generation of immune animals

For TRP-2 studies, 8- to 12-wk-old C57BL/6 mice were immunized in three s.c. sites with 10^6 irradiated (5000 cGy) B16 cells transfected with the gene for GM-CSF. B16 GM-CSF-secreting cells produced greater than 300 ng of GM-CSF/10^6 cells/24 h. Two weeks after the immunization, animals were challenged i.d. with 10^5 wild-type B16 tumor. Immune animals that resisted tumor growth were used 14–30 days after the tumor challenge. In some experiments, the p15E-expressing tumors MC38 or MCA 205 were used to generate anti-p15E CTL. MC38- and MCA205-immune animals were generated by vaccinating with live tumor admixed with 50 μg of *Corynebacterium parvum*, followed by surgical resection of the vaccination site 10–14 days later. Mice were then challenged with a tumorogenic inoculum of autologous tumor to identify successfully immunized animals.

Generation of primary CTL cultures

Spleens were harvested from appropriate animals and mechanically disrupted in the presence of ACK RBC lysis buffer (Biofluids). This suspension was than passed through 100-gauge sterile nylon mesh and washed three times in HBSS (Life Technologies). Splenocytes were then placed at 4–5 $\times$ 10^6 cells/well in 24-well plates (Costar, Cambridge, MA) in complete medium. Appropriate concentrations of each peptide were added directly to the culture. IL-2, 30 IU/ml (Chiron, Emeryville, CA), was added to cultures on the second and fourth days of culture. Nonviable cells were removed on day 6 by passage over a discontinuous lympholyte-M gradient (Accurate Scientific, Westbury, NY). Viable cells were washed and replated in fresh complete medium with IL-2 (30 IU/ml) at concentration of 10^6 cells/well. Primary cultures were tested between days 8 and 10.

Generation of CTL lines

Long-term CTL lines were produced by restimulation of primary cultures. Stimulators were generated by incubating fresh splenocytes in the presence of the different concentrations of peptide for 45 min. Cells were then washed three times and irradiated (2500 cGy). A total of 4 $\times$ 10^6 stimulator cells were then added to approximately 2 $\times$ 10^6 CTL/2-ml well. Fresh IL-2 was added 2 days after restimulation and replaced every 2–3 days. Repeat stimulation was performed every 7–10 days.

Cytokine release assays: IFN-γ or GM-CSF release assay

Briefly, primary cultures or long-term CTL lines were harvested and washed once and suspended at 10^6 cells/ml in complete medium. Peptide-pulsed target cells were generated by incubating 293K^b with the appropriate concentration of peptide for 45 min at room temperature. Target cells were then washed three times in HBSS and resuspended at 10^6 cells/ml in complete medium. Tumor cell targets were harvested from cell culture by trypsinization, washed twice, and resuspended at 10^6 cells/ml. Cytokine release assays were performed by incubating 10^5 effectors with 10^5 targets in 96-well microtiter plates (Costar). Supernatants were harvested from duplicate wells after 16–24 h and tested using an IFN-γ or GM-CSF ELISA (Endogen, Woburn, MA).

Adoptive transfer therapy model

Recipient C57BL/6 mice were inoculated with 3.5 $\times$ 10^5 B16 tumor cells in HBSS vial tail vein injection on day 0. On day 4, varying numbers of anti-TRP-2 CTL were administered i.v. and 6 $\times$ 10^4 IU of IL-2 were given i.p. three times a day for 3 days. On day 14, mice were sacrificed and the number of pulmonary metastases were enumerated in coded, blinded fashion. Differences in the number of metastases were analyzed by Wilcoxon Rank Sum test. All *p* values are two-tailed.

Results

Naive mice required higher concentrations of in vitro peptide to generate CTL than tumor vaccinated mice

We previously reported that TRP-2-reactive CTL could be generated from the splenocytes of naive animals after a single in vitro stimulation using 10^{-5}–10^{-6} M of TRP-$2_{180-188}$ peptide (6). In fact, because of the background precursor CTL (pCTL) activity, little difference in TRP-2 reactivity (as measured by IFN-γ release assay) was noted between animals immunized with whole tumor vaccines and naive animals. We hypothesized that the pCTL found

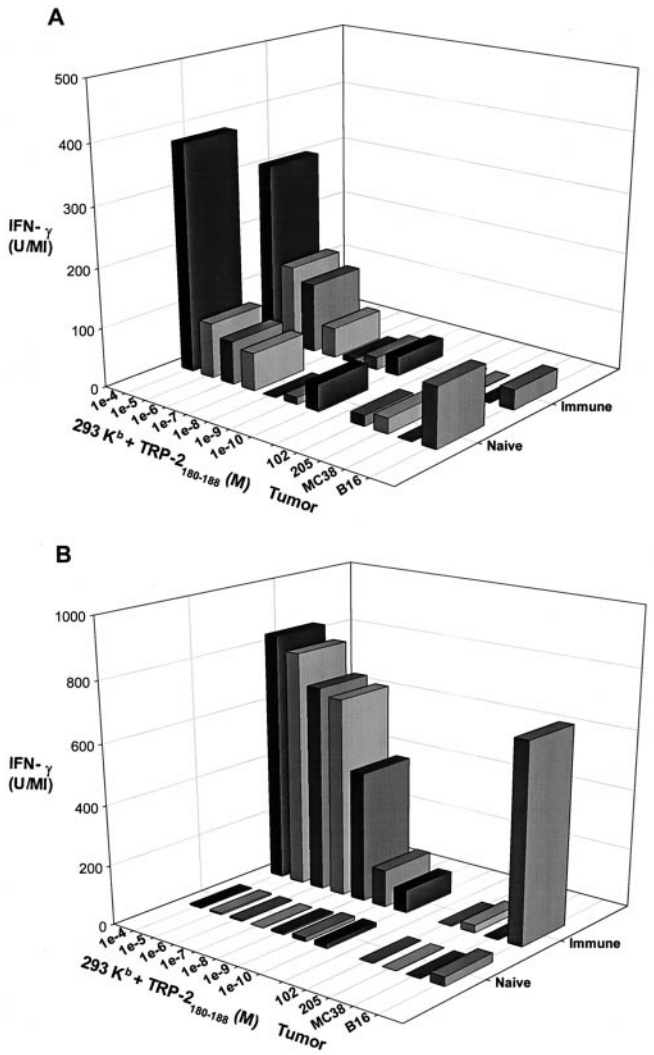

FIGURE 1. Generation of anti-TRP-2 CTL with high and low peptide concentrations. INF-γ release of primary CTL cultures (day 9) generated with either 10^{-5} M (*A*) or 10^{-9} M (*B*) TRP-$2_{180-188}$ peptide in response to 293K^b target cells pulsed with peptide and tumor targets. Both naive and immune animals generated low avidity CTL when 10^{-5} M of in vitro-stimulating peptide was used. Only immune animals were able to yield Ag-specific CTL when the concentration of in vitro-stimulating peptide was reduced to 10^{-9} M.

in naive animals were low avidity lymphocytes that would respond only to high concentrations of peptide in vitro. To test whether successfully vaccinated animals contained pCTL capable of being stimulated by lower concentration of in vitro-stimulating peptide, we compared the anti-TRP-2 reactivity between naïve animals and animals made immune to the B16 tumor through vaccination with B16 transfected with the gene for GM-CSF. It has previously been shown that this is an effective vaccination strategy resulting in nearly 80% of animals resistant to tumor rechallenge after a single vaccination (7). Splenocytes from two to three animals in each group were harvested, pooled, and cocultured with 10^{-5} or 10^{-9} M of the TRP-$2_{180-188}$ peptide as described in *Materials and Methods*. CTL were assayed on day 9 of culture for their ability to release IFN-γ in response to tumor and titered concentrations of the TRP-$2_{180-188}$ peptide pulsed onto 293K^b cells. As previously reported, both naive and vaccinated animals generated Ag-reactive CTL when their splenocytes were incubated with 10^{-5} M of in vitro-stimulating peptide (Fig. 1*A*). However, when the peptide

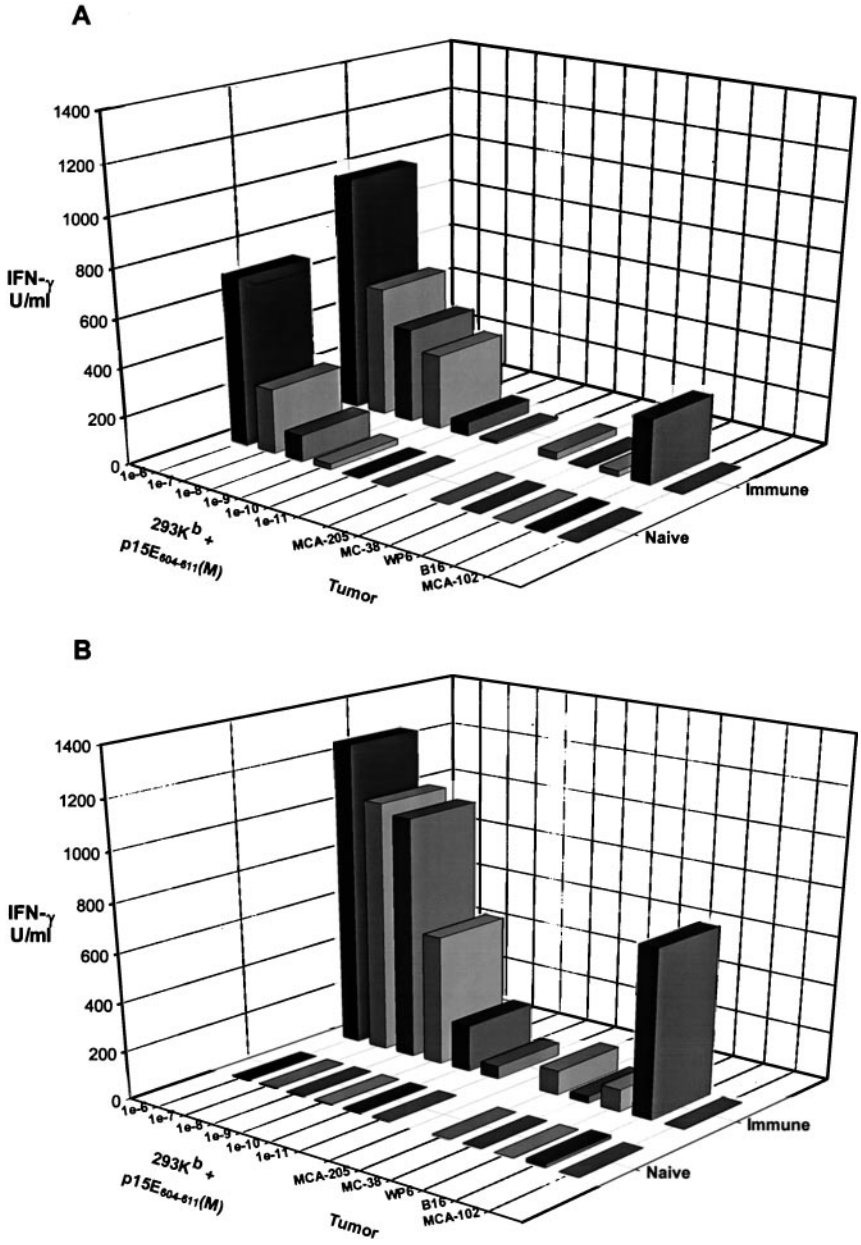

FIGURE 2. Generation of anti-p15E CTL with high and low peptide concentrations. INF-γ release of primary CTL cultures (day 9) generated with either 10^{-5} M or 10^{-9} M $p15e_{604-611}$ peptide in response to $293K^b$ cells pulsed peptide and tumor targets. As was true for TRP-2, both naïve and immune animals generated low avidity CTL when 10^{-5} M of in vitro-stimulating peptide was used *A*. Only immune animals were able to yield Ag-specific CTL when the concentration of in vitro-stimulating peptide was reduced to 10^{-9} M (*B*).

concentration used for in vitro stimulation was lowered to limiting concentrations (10^{-9} M), only vaccinated animals produced Ag-reactive CTL culture (Fig. 1*B*). We observed similar findings for another B16 tumor associated Ag recently characterized in our laboratory, p15E. In multiple experiments, splenocytes from naive animals consistently yielded reactivity to the p15E $aa_{604-611}$ epitope after a single in vitro stimulation using 10^{-5} to 10^{-6} M peptide (Fig. 2*A*). When the concentration of stimulating peptide was decreased to limiting conditions (10^{-9} M, Fig. 2*B*) only animals immune to the p15E-expressing tumor MCA205 were able to generate CTL. In most experiments, CTL generated with 10^{-5} M peptide (Figs. 1*A* and 2*A*) showed inferior recognition of $293K^b$ targets sensitized with low concentrations of the peptide Ag and weaker tumor reactivity when compared with CTL generated with 10^{-9} M peptide (Figs. 1*B* and 2*B*). This led us to investigate the optimal peptide concentration for activation of pCTL for these two Ags.

High concentrations of in vitro-stimulating peptide were detrimental to the generation of high avidity CTL: lower concentrations generated CTL with higher avidity

Splenocytes from at least two B16-immune animals were harvested, pooled, and cocultured with a broad range TRP-$2_{180-188}$ peptide concentrations (10^{-5}–10^{-10} M). Cultures were harvested and assayed (on day 9) for the ability to recognize B16 and the TRP-2-negative tumors MCA101 and MCA102, as well as $293K^b$ cells pulsed with titered concentrations of the peptide epitope. As can be seen in Fig. 3*A*, the highest in vitro peptide concentrations (10^{-5} M to 10^{-6} M) resulted in CTL that were only able to recognize targets pulsed with high concentrations of the peptide Ag (low avidity). Furthermore, these CTL demonstrated little or no release of IFN-γ in response to B16 tumor. In contrast, CTL cultures generated with lower concentrations of in vitro-stimulating peptide (10^{-7}–10^{-9} M) consistently showed higher avidity for peptide-pulsed targets and demonstrated superior recognition of

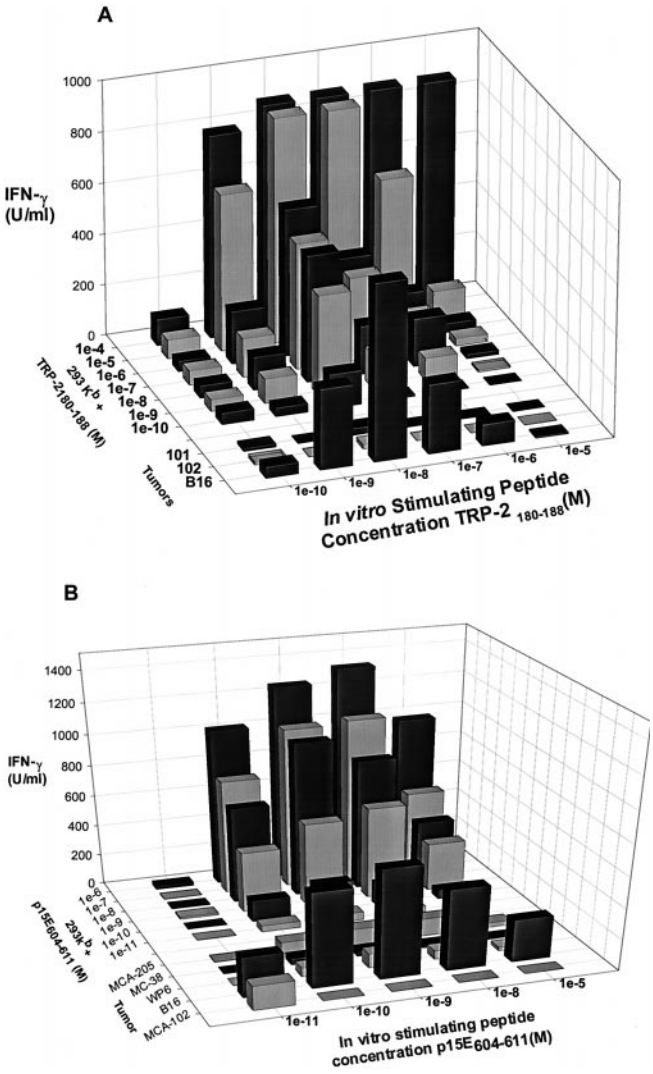

FIGURE 3. High concentrations of in vitro-stimulating peptide are detrimental to the generation of high avidity CTL. Splenocytes from B16-immune animals (*A*) or from animals immune to the p15E-expressing tumor MCA205 (*B*) were stimulated in vitro with titered concentrations of the appropriate peptide epitope. Primary cultures were tested (day 9) for the ability to release IFN-γ in response to tumor targets or to 293K^b cells pulsed with the appropriate autologous peptide. High concentrations of in vitro-stimulating peptide were detrimental to development of high avidity CTL for both these tumor-associated Ags. Results are representative of at least four independent experiments for each Ag.

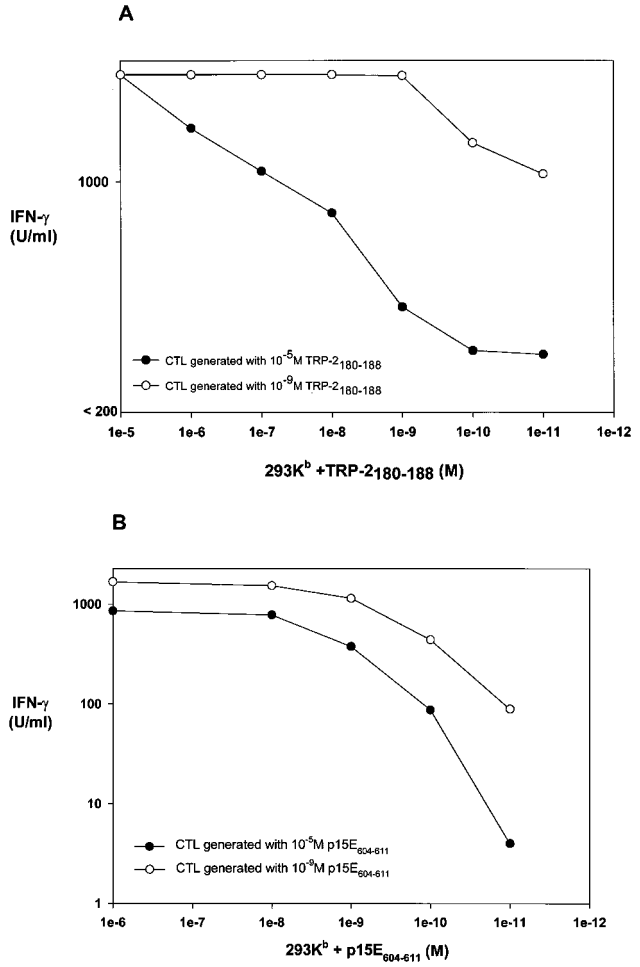

FIGURE 4. Selective expansion of high or low avidity CTL lines. CTL lines were generated by restimulation of primary cultures as described in *Materials and Methods*. TRP-2-specific CTL lines expanded over four in vitro stimulations with high concentrations of peptide (10^{-5} M) demonstrated low avidity for peptide-pulsed targets. CTL expanded in low concentrations (10^{-9} M) of stimulating peptide demonstrated high avidity for the peptide Ag (*A*). Similarly, high and low avidity CTL lines were able to be generated for the p15E Ag (*B*).

the B16 tumor. This same phenomenon was also observed for the $p15E_{604-611}$ Ag (Fig. 3*B*). High concentrations of in vitro-stimulating peptide (10^{-5} M) generated low avidity CTL cultures; lower concentrations of stimulating peptide (10^{-8}–10^{-9} M) generated CTL with high avidity. Again, as for TRP-2, higher avidity CTL cultures demonstrated superior recognition of p15E-expressing tumors. The inverse correlation between in vitro-stimulating peptide concentration and CTL activity was highly reproducible for both Ags.

Selective expansion of high and low avidity CTL

It has previously been shown by Alexander-Miller et al. (8) that CTL lines with high or low avidity for a virally-derived epitope could be selectively expanded by repetitive in vitro stimulation in bulk culture by controlling the concentration of stimulating peptide. We next investigated whether a similar phenomenon existed for our tumor-associated "self"-Ags. We selectively expanded CTL from tumor-immune animals with either high (10^{-5} M) or low (10^{-9} M) concentrations of the appropriate peptide. Cultures were restimulated every 7–10 days with a consistent concentration of peptide on irradiated splenocytes as described in *Materials and Methods*. As can be seen in Fig. 4*A*, CTL lines generated with limiting concentrations of $TRP\text{-}2_{180-188}$ peptide (10^{-9} M) for four in vitro stimulations demonstrated as much as 100-fold greater avidity for the epitope when compared with CTL generated with a high concentration (10^{-5} M) of peptide. Similarly, p15E-specific CTL lines expanded in a high concentration of peptide (10^{-5} M) demonstrated lower avidity for the Ag than those generated with low concentrations (10^{-9} M), with 4- to 20-fold higher peptide concentration required for $\frac{1}{2}_{maximal}$ IFN-γ release on multiple assays (Fig. 4*B*). For both Ags, the ability to recognize targets cells sensitized with low concentrations of peptide correlated with better in vitro tumor recognition (data not shown). There was no difference in growth rate or total number of cells generated under each of these peptide concentrations.

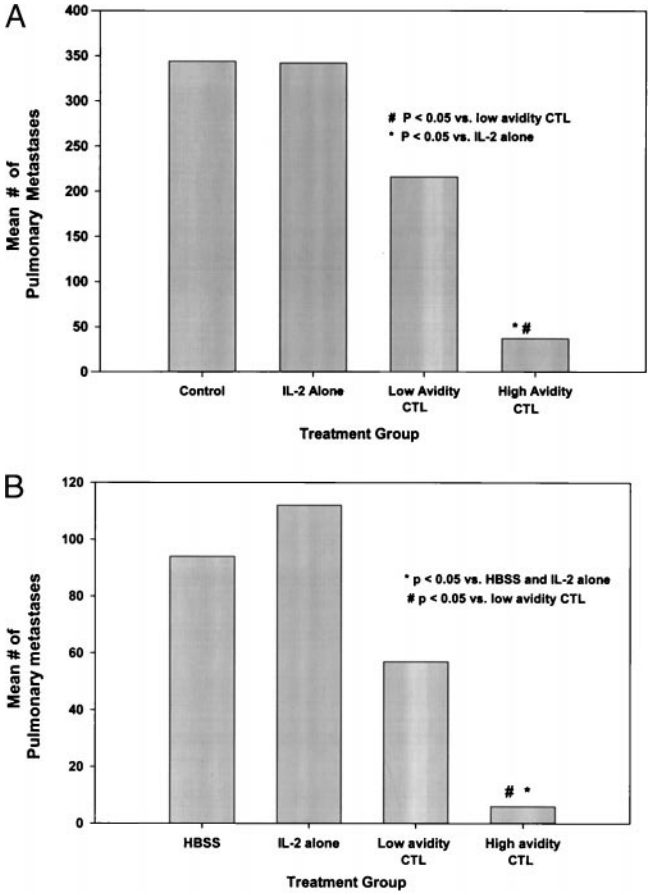

FIGURE 5. Treatment of day 4 B16 pulmonary metastases by high and low avidity CTL lines. A total of 3×10^6 high and low avidity TRP-2-specific CTL were adoptively transferred into C57BL/6 mice bearing day 4 pulmonary metastases as described in *Materials and Methods*. Fourteen days later mice were sacrificed an the number of pulmonary metastases were enumerated in a coded, blinded fashion. Two-tailed *p* values were calculated using the Wilcoxon Rank Sum test. In a separate experiment, 7×10^4 high or low avidity p15E-specific CTL were used to treat 4-day established B16 pulmonary metastases (*B*). For both Ags, only the high avidity CTL effectively reduced the number of pulmonary metastases. Results are representative of two independent experiments for each Ag.

High avidity CTL cultures, generated with low concentrations of peptide, were more effective in treating established B16 pulmonary metastases

We next asked if better in vitro avidity of CTL correlated with enhanced in vivo antitumor efficacy. To test this, C57BL/6 mice were injected via the tail vein with 3×10^5 B16 tumor (which expresses high levels of both TRP-2 and p15E), and 4 days later varying numbers of high or low avidity CTL (after four in vitro restimulations) were given via tail vein. IL-2 (60,000 IU) was given i.p. three times a day for 3 days after adoptive transfer. Fourteen days later the animals were sacrificed and the total number of pulmonary metastases were enumerated in a blinded coded protocol. As can be seen in Fig. 5*A* (TRP-2) and 5*B* (p15E), only the high avidity CTL cultures generated with the low concentrations of in vitro-stimulating peptide effectively reduced the pulmonary metastases.

Discussion

In this study, we examined the self-reactive CTL for two recently described nonmutated tumor associated Ags for the B16 murine melanoma: TRP-2 and p15E (although the p15E protein is a retroviral envelope protein, the retroviral genome is incorporated into the germline of the C57BL/6 strain, and it is expressed, nonmutated, in multiple syngeneic tumors of this strain.). We had previously observed that CTL reactive to these two self-Ags could be generated from naive animals by a single in vitro peptide stimulation. The significance of pCTL to these self-Ags in naive animals is not fully understood; however, it is possible that they represent CTL specific for environmentally encountered Ags cross-reacting with TRP-2 and p15E with low avidity. Similar mechanisms have been postulated to explain the existence of pCTL to MART-1 human melanoma Ag in normal volunteers (9). We postulated that lower concentrations of in vitro-stimulating peptide would better discriminate between naive and tumor-immune animals by eliminating activation of these cross-reactive, low avidity, pCTL. Our experimental results confirm that splenocytes from naive animals produce Ag-reactive CTL cultures only at high concentrations of in vitro-stimulating peptide. Immune animals, however, contained highly avid effector CTL, which were able to be activated by extremely low concentrations of Ag. In fact, by titrating the in vitro stimulating peptide concentration, we were able to distinguish between a variety of Ag-reactive states. A hierarchy of reactivity based on the ability of splenocytes to respond to increasingly lower concentrations of in vitro-stimulating peptide was consistently observed: immune animals > tumor-bearing > peptide immunized > naive (our unpublished observations). Somewhat surprisingly, we observed that high concentrations of in vitro stimulating peptide had a detrimental effect on the avidity and antitumor reactivity of CTL from immune mice. Several groups have reported that high concentrations of in vitro-stimulating peptide can be detrimental to T cells (10–12). Iezzi et al. (11) recently demonstrated that transgenic $CD4^+$ T cells specific for hemagglutinin Ag underwent activation-induced cell death after prolonged exposure to Ag in vitro. Alexander-Miller et al. (12) have demonstrated that $CD8^+$ CTL can also undergo inhibition of proliferation and cell death in response to supraoptimal peptide/MHC concentrations in vitro. Moreover, susceptibility to activated cell death in this system was directly correlated with the avidity of the CTL (12). The "artificially" high concentrations of peptide in vitro presumably cause activation-induced cell death of Ag-specific T cells. However, T cells with low avidity for the peptide epitope are less susceptible to "overstimulation" and thus predominate in cultures generated with high concentrations of peptide. High or low avidity CTL for a viral Ag could be generated by controlling the concentration of in vitro stimulating peptide, and higher avidity in vitro correlated with better in vivo antiviral activity of these CTL when adoptively transferred (8). Our data for CTL generation against tumor-associated, self-Ags support these findings. High avidity CTL (generated with low concentrations of peptide in vitro) demonstrated superior clearance of B16 pulmonary metastases when adoptively transferred into tumor-bearing animals. Although in previous work, CTL generated with high (10^{-6} M) peptide concentrations could reduce 3-day-old pulmonary metastases, here they were ineffective against a greater tumor burden. Although little data on the cytotoxicity of these cultures was obtained, the differences in avidity demonstrated here using cytokine release measurements were corroborated by differences in cytotoxicity whenever cultures were simultaneously assayed (data not shown). The release of IFN-γ by CTL as an indicator of immune recognition has previously been shown to correlate well with in vivo antitumor activity (13) and these data again demonstrates this finding.

These observations may have important applications to clinical therapy. First, they suggest that the method used to generate CTL

in vitro directly affect their in vivo efficacy. This could be important to ongoing adoptive immunotherapy trials in which PBMCs specific for defined tumor-associated epitopes are expanded in vitro by repetitive peptide stimulation prior to reinfusion. Expansion under optimal peptide concentrations might generate CTL with higher avidity for the Ag. Our observations suggest that this higher avidity correlates well with better in vitro and in vivo antitumor activity. Similar observations (higher avidity is critical to optimal recognition of tumor) have been made with human CTL clones specific for $gp100_{209-217}$ melanoma-associated Ag (14). Using high efficiency cloning techniques, clones with a broad range of avidity for the Ag have been identified from that PBMC of patients immunized with a modified synthetic peptide encoding the Ag. The avidity of these clones correlated closely with their in vitro tumor reactivity. These observations have formed the basis for several new clinical trials investigating the adoptive transfer of large numbers of a high avidity $CD8^+$ clone. Our observations also suggest that methods of in vivo vaccination that activate large numbers of low avidity CTL may not be useful if these CTL cannot recognize physiological amounts of endogenously processed and presented tumor Ag. (Thus far we have not been able to demonstrate similar phenomena in human PBMC from immunized patients.) In fact, these data further suggest that high concentrations of Ag in vivo may be detrimental to the generation of highest avidity CTL. Others have reported induction of epitope-specific tolerance and enhanced tumor growth in vivo after immunization with high concentrations of peptide epitopes (15, 16).

In summary, our data suggest that the ability to generate an effective antitumor response to self nonmutated Ags may be limited by the quality (avidity) of the CTL generated. Furthermore, these observations demonstrate the means, and the need, for more precise characterization of the autoreactive CTL generated by vaccination against self tumor-associated Ags.

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