# Cutting Edge: Passive But Not Active CD8$^+$ T Cell-Based Immunotherapy Interferes with Liver Tumor Progression in a Transgenic Mouse Model[1]

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**To evaluate tumor immunotherapies, we used transgenic mice that harbor a progressive liver tumor associated with the expression of the SV40 large tumor T oncoprotein (SV40-T). To induce "self" tumor Ag-specific CD8$^+$ T cells, mice were injected with an immunodominant SV40-T CTL epitope mixed with a heterologous helper peptide. Despite repeated injections, this vaccine failed to raise a tumor-specific CD8$^+$ T cell response that was efficient enough to counteract tumors. Although coimmunization with SV40-T CTL epitope and heterologous helper peptide efficiently recruited the respective Th cells, only low-avidity SV40-T-specific CD8$^+$ T cells were activated. Furthermore, major alterations in SV40-T-specific B and Th cell responses were characterized. In contrast, transfers of higher-avidity CTLs specific for the same SV40-T epitope were effective in counteracting tumors. These results suggest that passive therapies targeted to self tumor Ag may be more suitable than active immunization in the treatment of spontaneous tumors.** ***The Journal of Immunology*****, 1998, 161: 5133–5137.**

The identification of antigenic peptides from processed tumor Ag (1) has led to new insights in T cell cancer therapy by active immunization or adoptive CTL transfer (2–4). The design of tumor vaccines may be hampered by the difficult task of reversing immune unresponsiveness toward tumor Ag, which is most commonly self Ag in human tumors (5).

In the present study, we took advantage of a transgene-encoded viral oncoprotein which is also a "self" tumor Ag that accumulates in tumor cells. Transgenic mice of the antitrobin III-SV40T (ASV-B)[3] lineage develop a progressive hepatocellular carcinoma (HCC)[3] that is related to the hepatocyte-restricted expression of SV40 large tumor T oncogene (SV40-T) (6). The expression of SV40-T is placed under the control of the antithrombin III liver-specific promoter, which is a differentiation-like tumor Ag such as tyrosinase, Melan/MART-1, or gp100 in human melanomas (1). This transgenic model is probably more representative of spontaneous tumorigenesis than tumor transplantation. In the present study, we explored the effectiveness of CD8$^+$ T cell-based immunotherapies on tumor progression by comparing peptide immunization and adoptive transfers of CTLs.

## Materials and Methods

### *Mice*

Transgenic (C57BL/6 × DBA/2) ASV-B mice carried the SV40 early genes controlled by the antithrombin III liver-specific promoter on the Y chromosome and succumbed to HCC before 36 wk of age (6, 7). These mice were backcrossed with C57BL/6 mice (>25 generations).

### *Peptides, Ab reagents, and cell lines*

SV40-T$_{205–215}$, SV40-T$_{223–231}$, SV40-T$_{404–411}$, and SV40-T$_{488–497}$ CTL epitopes of SV40-T Ag, Gag$_{390–398}$ CTL epitope of the HIV gag protein, nucleoprotein 366–374 (NP$_{366–374}$) of the influenza virus nucleoprotein CTL epitope, and hepatitis B virus core protein (HBVc) 128–140 helper epitope peptides were obtained from Neosystem (Strasbourg, France). The anti-mouse IFN-γ (clones R4-6A2 and XMG1.2) Abs were obtained from PharMingen (San Diego, CA). The SV40-T-specific PAb419 Ab was provided by Dr. E. May (Commissariat à l'Energie Atomique, Fontenay-aux-Roses, France). EL4 is a C57BL/6 T cell lymphoma. The culture medium used was RPMI 1640 medium (Life Technologies, Gaithersburg, MD), 10% FCS, 20 mM HEPES, 2 mM glutamine, antibiotics, and $5 \times 10^{-5}$ M 2-ME (Sigma, St. Louis, MO).

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[3] Abbreviations used in this paper: ASV-B, antitrombin III-SV40T; HCC, hepatocellular carcinoma; SV40-T, SV40 large tumor T oncogene; NP, nucleoprotein; HBVc, hepatitis B virus core protein; ELISPOT, enzyme-linked immunospot; SFC, spot-forming cells.

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*Peptide immunization with CTL epitopes and in vitro induction of CTL lines*

Mice were s.c. injected in the tail with 50 $\mu$g of CTL epitope mixed with 140 $\mu$g of $HBVc_{128-140}$ helper epitope peptides (8) in IFA. After 11 days, draining lymph node cell responses were tested ex vivo by IFN-$\gamma$ enzyme-linked immunospot (ELISPOT). To induce CTL lines, these cells were stimulated for 6 days with irradiated syngeneic LPS/dextran sulfate-activated lymphoblasts that had been pulsed with $10^{-5}$ M peptide (9) and were maintained weekly with peptide-pulsed C57BL/6 irradiated splenocytes. Human rIL-2 (10 U/ml, Boehringer Mannheim, Mannheim, Germany) was added every 3 days. Murine rIL-7 (10 ng/ml, Biosource International, Camarillo, CA) was added for the first stimulation.

*ELISPOT assay for single-cell IFN-$\gamma$ secretion*

The ELISPOT assay for detecting epitope-specific IFN-$\gamma$-secreting T cells was adapted from Miyahira et al. (10). Nitrocellulose microplates (Millipore, Bedford, MA) were coated with 3.5 $\mu$g/ml anti-mouse IFN-$\gamma$ (R4-6A2). A total of $5 \times 10^5$-$1.8 \times 10^4$ lymph node cells were tested in the presence of 30 U/ml human rIL-2. $CD8^+$ T cells were stimulated with $10^5$ irradiated MHC class II-negative EL4 cells pulsed (or not) with $10^{-5}$ M peptide. Th cells were activated by the addition of 30 $\mu$g/ml $HBVc_{128-140}$ peptide. After 24 h, the plates were washed, incubated with 3.5 $\mu$g/ml biotinylated anti-mouse IFN-$\gamma$ (XMG1.2), and subsequently incubated with alkaline phosphatase-labeled extravidin. After adding chromogenic alkaline phosphatase conjugate substrate (Bio-Rad, Hercules, CA), IFN-$\gamma$ spot-forming cells (SFC) were counted using a stereomicroscope.

*Immunization with recombinant SV40-T (rSV40-T) for analysis of Th cell responses*

Mice were initially injected s.c. in the tail and footpads with 10 $\mu$g of rSV40-T (Molecular Biology Resources, Milwaukee, WI) in CFA; after 2 wk, these animals were injected with 5 $\mu$g of rSV40-T in IFA. After 10 days, spleen cells ($5 \times 10^5$/well) were incubated with 0.1–10 $\mu$g/ml rSV40-T or 2.5 $\mu$g/ml Con A in culture medium containing 1% normal mouse serum and 2% FCS. After 24 and 48 h, supernatant was removed to test effector-released IL-2 and IL-4 with the CTL-L2 and CT4-S bioassays.

*Immunotherapy by peptide immunizations or CTL transfers*

ASV-B transgenic mice (7–8-wk-old) received three monthly peptide immunizations of SV40-$T_{223-231}$ mixed with $HBVc_{128-140}$ epitope peptides and were sacrificed 2 wk later. Negative controls were immunized with the irrelevant $D^b$-restricted CTL epitopes $Gag_{390-398}$ or $NP_{366-374}$. Alternatively, ASV-B transgenic mice (15–23 wk-old) were injected i.v. three times (days 1, 7, and 14) with $2 \times 10^6$ CTL line cells specific for SV40-$T_{223-231}$ or $NP_{366-374}$ (irrelevant epitope) and were sacrificed 7 days later. The CTL lines were from peptide-immunized nontransgenic C57BL/6 males and were injected just before the weekly restimulation of the CTL lines. Effector CTL functions were tested for specific lytic activity and IFN-$\gamma$ and TNF secretion. In treated mice, the modulation of tumor weight was calculated relative to nontreated, age-matched ASV-B transgenic mice. Liver histology was analyzed on hematoxylin and eosin-stained frozen sections. The SV40-T expression level was assessed by Western blot. Two independent liver samples for each mouse were treated for total protein extraction. Proteins (100 $\mu$g) were separated by SDS-PAGE on 10% acrylamide gels and transferred onto nitrocellulose membranes. Blots were revealed using SV40-T-specific mAb PAb419 of the IgG2b isotype and rabbit anti-mouse IgG2b (PharMingen).

## Results and Discussion

We tested the efficiency of $CD8^+$ T cell-based therapies that are specific for self tumor Ag epitopes. Four H-$2^b$-restricted SV40-T CTL epitopes have been described previously in C57BL/6 mice (11–13). SV40-$T_{205-215}$, SV40-$T_{223-231}$, and SV40-$T_{404-411}$ are codominant epitopes (12, 13), whereas SV40-$T_{488-497}$ is a subdominant epitope (13). Active therapy was targeted to a $D^b$-restricted CTL-dominant epitope mapping to the SV40-$T_{223-231}$ sequence. In our model, some of the four SV40-T CTL epitopes were found to be endogenously presented by three HCC cell lines derived from transgenic mice; SV40-$T_{223-231}$ was the most efficiently presented epitope by the HCC cell lines (14). Transgenic mice were immunized with

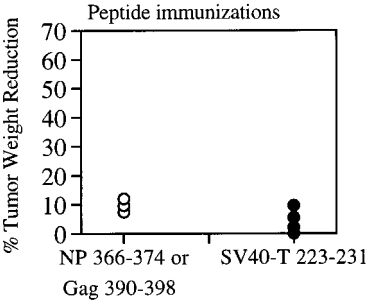


**FIGURE 1.** Absence of tumor weight reduction in peptide-immunized ASV-B transgenic mice. Transgenic mice were treated with three consecutive monthly injections of SV40-$T_{223-231}$ ($n = 5$) mixed with $HBVc_{128-140}$ helper peptide, of irrelevant CTL epitopes $NP_{366-374}$ ($n = 2$), or of $Gag_{390-398}$ ($n = 1$). Mice were 22 or 23 wk old when sacrificed. Actual values of liver weights were 6.4, 6.6, and 6.7 g (immunization with irrelevant CTL epitopes); 6.6, 6.9, 7.1, 7.2, and 7.6 g (immunization with SV40-$T_{223-231}$); or 7.3 $\pm$ 0.37 g (nontreated, age-related transgenic mice, $n = 7$).

SV40-$T_{223-231}$ by three monthly injections that began before the macroscopic tumor growth phase. The I-$A^b$-restricted helper peptide $HBVc_{128-140}$ was included in the immunizing formulation as described previously (8, 9). We observed neither reduction in liver tumor size (Fig. 1) nor major modifications of HCC histology in immunized mice (data not shown). Analysis of their liver SV40-T expression failed to demonstrate an alteration of tumor Ag expression (data not shown). Thus, repeated injections of an SV40-T CTL epitope peptide, even when supplemented with a potent helper peptide, failed to interfere with tumor progression in ASV-B transgenic mice.

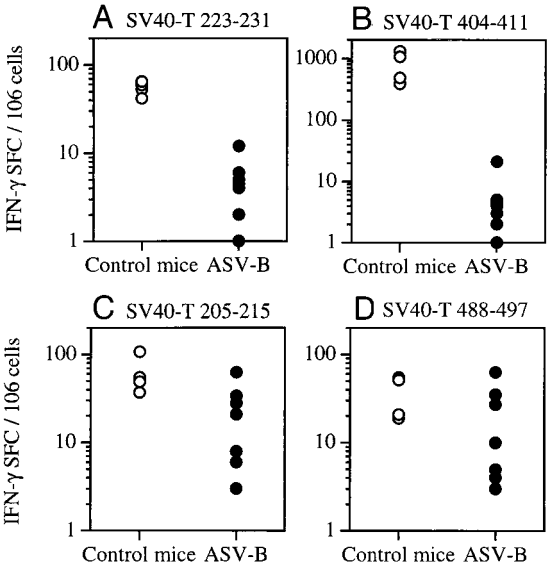


**FIGURE 2.** Analysis of SV40-T-specific $CD8^+$ T cell repertoire in ASV-B transgenic (●) and control (○) mice. Transgenic ($n = 7$) or control (nontransgenic ASV-B female mice, $n = 4$) mice were immunized with the pool of SV40-T CTL epitopes (SV40-$T_{205-215}$, SV40-$T_{223-231}$, SV40-$T_{404-411}$, and SV40-$T_{488-497}$) mixed with $HBVc_{128-140}$ helper peptide. Epitope-specific $CD8^+$ T cell responses were assayed ex vivo by IFN-$\gamma$ ELISPOT. Uncultured lymph node cells were exposed for 24 h to EL4 cells pulsed with individual SV40-T CTL epitopes. Results are expressed as numbers of IFN-$\gamma$ SFC per $10^6$ cells; the SD between replicates was <10%. The background values (always <10) obtained in the absence of peptide were subtracted from the values obtained with Ag stimulation.

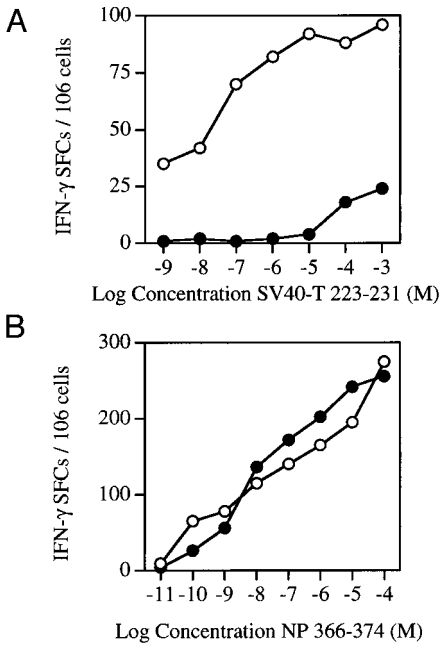


**FIGURE 3.** Comparison of $CD8^+$ T responses in 8-wk-old ASV-B transgenic (●) and control (○) mice. *A*,Transgenic or control mice ($n$ = three per group) were immunized with SV40-$T_{223–231}$ and $HBVc_{128–140}$. *B*, Transgenic ($n$ = four per group) or control mice ($n$ = three per group) were immunized with $NP_{366–374}$ and $HBVc_{128–140}$. Epitope-specific T cell responses were assayed ex vivo by IFN-γ ELISPOT. Uncultured lymph node cells were exposed for 24 h to EL4 cells pulsed with SV40-$T_{223–231}$ ($10^{-9}$–$10^{-3}$ M) (*A*) or to EL4 cells pulsed with $NP_{366–374}$ ($10^{-11}$–$10^{-4}$ M) (*B*). Results are expressed as described in Fig. 2.

To assess active immunization failure, we analyzed the induction of SV40-T-specific $CD8^+$ T cells in transgenic mice immunized with a mixture of the four SV40-T CTL epitope peptides. Vaccination with CTL epitope peptides in IFA has been reported to be protective against viral infections (15, 16) or tumor transplantations (3). However, in this progressive tumor, such an immunization protocol failed to induce detectable SV40-T-specific $CD8^+$ T cell responses (data not shown). In an attempt to amplify in vivo $CD8^+$ T cell responses, mice were coimmunized with a potent helper peptide, $HBVc_{128–140}$ (8, 9). Specific $CD8^+$ T cells were quantified ex vivo by an IFN-γ ELISPOT conducted on uncultured lymph node cells. The number of SV40-$T_{223–231}$- and SV40-$T_{404–411}$-specific $CD8^+$ T cells was dramatically reduced in 8-wk-old immunized transgenic mice compared with nontransgenic control littermate mice (Fig. 2, *A* and *B*). It is noteworthy that these responses were further reduced in >20-wk-old transgenic mice (terminal phases of HCC; data not shown). Nevertheless, the presence of SV40-$T_{205–215}$- and SV40-$T_{488–497}$-specific $CD8^+$ T cells could be detected in half of the transgenic mice (Fig. 2, *C* and *D*). Therefore, transgenic mice displayed various degrees of alteration of the SV40-T-specific $CD8^+$ T cell repertoire affecting mainly the dominant SV40-T CTL epitopes, previously defined in C57BL/6 mice (12, 13). Indeed, two of the three codominant SV40-T CTL epitopes were profoundly tolerogenic, whereas the subdominant epitope was partially tolerogenic. Of note, we observed no clear correlation between the induction of tolerance and the hierarchy of the CTL epitopes previously defined for their presentation by HCC tumor cell lines, since only one (SV40-$T_{223–231}$) of the two tolerogenic dominant epitopes (SV40-$T_{223–231}$ and SV40-$T_{404–411}$) was found to be presented in vitro (14).

Table I. *SV40-T-specific Th cell responses by spleen cells from ASV-B transgenic or control mice*

| Mice[a] | Age (wk) | IL-2 Production[d] | IL-4 Production[d] |
|---|---|---|---|
| Transgenic mice | 8 | 1.0 | ND |
| | 9 | 0.8 | 0.9 |
| | 8 | 1.1 | 1.0 |
| | 18 | 1.6 | 0.9 |
| | 18 | 1.2 | 0.8 |
| Control mice | 8[b] | 7.3 | ND |
| | 8[b] | 2.2 | 4.2 |
| | 18[b] | 7.5 | 10.5 |
| | 8[c] | 7.4 | 5.8 |
| | 19[c] | 8.7 | 0.6 |

[a] SV40-T-specific Th cell responses were tested in rSV40-T-immunized ASV-B transgenic or control mice [b]nontransgenic ASV-B female mice or [c] C57BL/6 male mice). Results are shown at 3 μg/ml rSV40-T.

[b] IL-2 secretion was assayed after 24 h in culture with the CTL-L2 bioassay, and IL-4 secretion was assayed after 48 h in culture with the CT4-S bioassay. Results show the proliferation of CTL-L2 or CT4-S cells ($\times 10^{-3}$ cpm) obtained with specific Ag stimulation. Background CTL-L2 or CT4-S cell proliferations obtained without Ag stimulation were under 800 cpm. Standard curves using 0.03100 U/ml murine rIL-2 (3,560 to 189,590 cpm) or murine rIL-4 (1,160 to 86,230 cpm) were completed to standardize the cytokine-dependent proliferation of CTL-L2 or CT4-S cells. Data are expressed as the arithmetic mean of three independent replicates; SD was <15%.

We subsequently wondered whether the altered SV40-T-specific $CD8^+$ T cell response was linked to a multispecific immunosuppression of Th or $CD8^+$ T cell responses that could have been exerted by tumor cells. Both transgenic and control mice also responded to $HBVc_{128–140}$ by specific T cell proliferation and IFN-γ and IL-2 secretion but not by IL-4 secretion (data not shown), suggesting that these mice could mount equivalent Th1 cell responses to nonself $HBVc_{128–140}$ helper peptide.

In addition, no IL-4- or TNF-secreting SV40-$T_{223–231}$-specific $CD8^+$ T cells were detected ex vivo in transgenic mice immunized with SV40-$T_{223–231}$ mixed with $HBVc_{128–140}$ (data not shown). We subsequently assessed the avidity of SV40-$T_{223–231}$-specific $CD8^+$ T cells by ex vivo IFN-γ ELISPOT using stimulation with a range of Ag concentrations (Fig. 3*A*). Control mice still had a significant proportion of $CD8^+$ T cells that were activated with $10^{-9}$ M SV40-$T_{223–231}$. Strikingly, SV40-$T_{223–231}$-specific $CD8^+$ T cells from transgenic mice required $10^5$-fold more peptide to reach detectable activation levels. On the contrary, control and transgenic mice immunized with the heterologous $NP_{366–374}$ influenza epitope mounted $CD8^+$ T cell responses that were comparable in both number and avidity (Fig. 3*B*). Altogether, these results suggest that alterations in high-avidity $CD8^+$ T cells are specific for SV40-$T_{223–231}$ and are linked to self tumor Ag-specific deletion or anergy rather than to immune deviation. In vitro, it was nevertheless possible to expand the reduced number of specific $CD8^+$ T cells from immunized transgenic mice. In most instances, one round of restimulation was sufficient to induce SV40-$T_{223–231}$-specific transgenic CTL lines displaying lymphokine production and lytic activities comparable with control CTL lines (data not shown). These observations support the relevance of an ex vivo analysis of the T cell repertoire, as reported in other studies (17).

To further explore self tumor Ag-specific tolerance in transgenic mice, B and Th cell responses were assayed in mice immunized with rSV40-T protein. In control mice, the activation of spleen cells by rSV40-T was demonstrated by the specific production of IL-2 and IL-4 (Table I) in the absence of significant cell proliferation (data not shown). Immunized transgenic

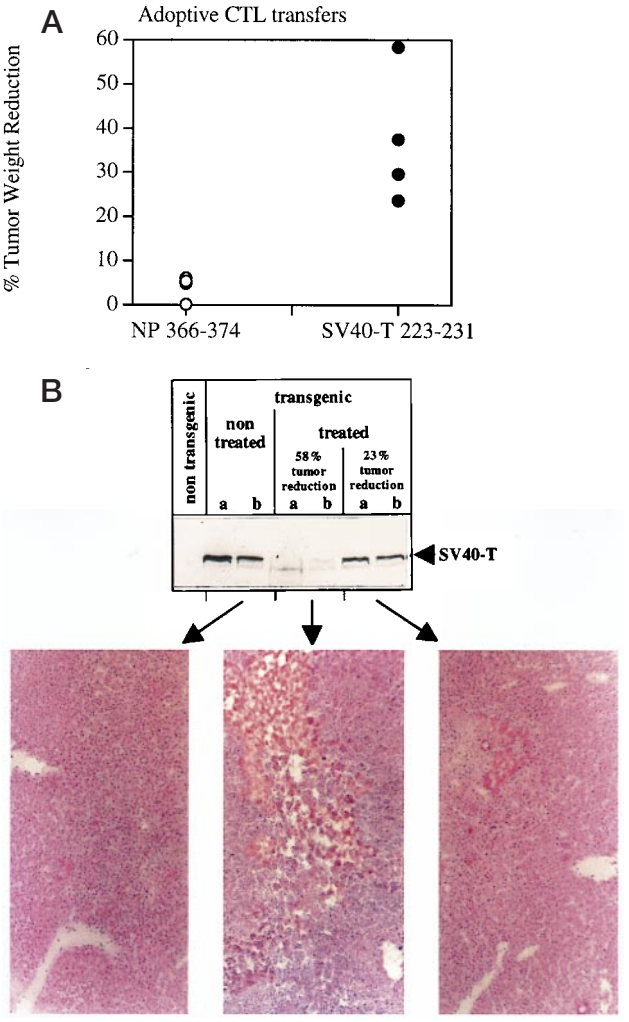


**FIGURE 4.** Reduction of tumor weight in ASV-B transgenic mice treated by CTL adoptive transfers. *A*, Transgenic mice received three consecutive weekly transfers of CTL line cells specific for SV40-$T_{223–231}$ ($n$ = 4) or for the irrelevant epitope $NP_{366–374}$ ($n$ = 4). Mice were aged 20, 25, or 27 wk when sacrificed. Actual values of liver weights were 6.6 and 6.4 g (20 wk), 7.0 g (25 wk), or 7.7 g (27 wk) for mice treated by transfers of irrelevant CTLs; 4.2 and 4.8 g (20 wk) or 3.3 and 6.0 g (27 wk) for mice treated by transfers of SV40-$T_{223–231}$-specific CTLs; and 6.8 ± 0.2 g (20 wk, $n$ = 6), 7.4 ± 0.5 g (25 wk, $n$ = 8), or 7.9 ± 0.6 g (27 wk, $n$ = 8) for nontreated, age-related transgenic mice. *B*, Liver SV40-T expression level and histology of the two transgenic mice displaying a 58% and 23% reduction, respectively, of tumor weight upon transfers of SV40-$T_{223–231}$-specific CTLs. SV40-T expression was assessed on protein extracts from two independent liver samples. Positive controls were from nontreated, age-matched transgenic mice; negative controls were from nontransgenic ASV-B female mice. A histologic examination (×40) of hematoxylin and eosin-stained frozen liver sections showed large foci of necrosis. Controls were nontreated, age-matched transgenic mice.

mice (8 and 18 wk old) did not show detectable SV40-T-specific Th cell responses (Table I), although they did display normal responses to Con A (data not shown). The addition of IL-1$\alpha$, a costimulator of Th2 cell proliferation (18), to cell culture could not revert this unresponsiveness (data not shown). There were major concomitant qualitative and quantitative reductions in SV40-T-specific B cell responses in transgenic mice (data not shown). Thus, SV40-T Ag is most probably presented in vivo, leading to early tolerance of SV40-T-specific immune cells. Various mechanisms may account for self-tolerance (19); in particular, the expression of SV40-T Ag during perinatal life (6) might shape the transgenic immune repertoire (19). However, we found that a number of tumor-specific $CD8^+$ T cells escape tolerance, most probably due to their lower avidity. Interestingly, such regulations may affect the specific repertoire for self tumor Ag (20).

It has been reported recently that the activation of low-avidity $CD8^+$ T cells specific for a self tumor Ag epitope can protect against tumor cell challenge in mice (21). In our study, the activation of low-avidity $CD8^+$ T cells upon peptide immunization failed to counteract tumor progression. This finding could be due to defects in either effector T cells or in tumor cell sensitivity to immune effectors. To test the second possibility, we treated transgenic mice that had already fully developed HCC by adoptive CTL transfers. SV40-$T_{223–231}$- or control $NP_{366–374}$-specific CTL lines that had been established from nontransgenic syngenic C57BL/6 male mice were checked for specific lytic activity and IFN-$\gamma$ or TNF secretion before transfers (data not shown). Transfers of SV40-$T_{223–231}$-specific CTLs reduced tumor weight by 23–58% (Fig. 4*A*). There was also a major reduction of the SV40-T liver expression level of the mouse displaying a 58% reduction in tumor weight; massive liver necrosis was observed (Fig. 4*B*). In the mouse having a 23% reduction in tumor weight, necrosis was limited to small areas, whereas no significant reduction in SV40-T expression was detected (Fig. 4*B*). In contrast, transgenic mice treated with $NP_{366–374}$-specific CTLs did not show any of these modifications (data not shown). We could not transfer SV40-$T_{223–231}$-specific CTL lines established from immunized transgenic mice because of their reduced ability to proliferate in vitro (data not shown). Nevertheless, the results obtained attested to both the antigenicity and sensitivity of liver tumor cells in vivo to SV40-$T_{223–231}$-specific effector CTLs.

Both intrinsic selection of the immune repertoire and effects of tumors may constitute drawbacks to immunotherapies targeted at tumor Ag. Using a transgenic model, we found that adoptive transfer of self tumor Ag-specific CTLs was more suited to interfering with tumor development than active peptide immunization. This difference could be linked to major alterations in the tumor-specific immune repertoire that are likely to occur in a similar fashion in spontaneous tumors (1, 20). Thus, a selection of autologous $CD8^+$ T cells appears necessary to elicit optimal tumor Ag-specific effector functions for adoptive transfer in patients. This selection could be assessed during the necessary phase of in vitro expansion or directly in vivo by adjuvant treatments (22).

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