Regression of Established Liver Tumor Induced by Monoepitopic Peptide-Based Immunotherapy[1]

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Most types of cancer are difficult to eradicate, and some, like hepatocellular carcinoma, are almost always fatal. Among various interventions to improve the survival of patients with cancer, immunotherapy seems to hold some promises. However, it requires relevant animal models for preclinical development. In this study we report a new and relevant experimental model where liver tumors grow inside a nontumoral parenchyma of adult mice. This model is based on the intrasplenic injection in syngeneic recipient mice of hepatocytes from transgenic mice expressing SV40 large T oncogene specifically in the liver. Using this model where no apparent spontaneous cellular immune response was observed, immunization using a single injection of monoepitopic SV40 T Ag short peptide was sufficient to provoke liver tumor destruction, leading rapidly to complete remission. Tumor regression was associated with the induction of a long-lasting $CD8^+$ T cell response, observed not only in the spleen but also, more importantly, in the tumoral liver. These results show the efficacy of peptide immunotherapy in the treatment of liver cancer. ***The Journal of Immunology,*** **2004, 173: 4882–4888.**

With an estimated incidence of about one million cases, hepatocellular carcinoma (HCC),[4] is one of the most common fatal cancers in the world (1). HCC in humans have a very poor prognosis, and current treatment efficacy is limited by low response rates, severe toxicity, and high recurrence rates, resulting in a mean survival time of only 6 mo (2). Thus, novel strategies, such as immunotherapy, are needed to improve the survival of patients with HCC. Recent progress in the molecular identification of tumor-associated Ags (3) and understanding the mechanisms of Ag presentation and their recognition by T lymphocytes (4) provide a valuable and promising basis for immunotherapeutic interventions. The crucial role of CTL in tumor rejection has been assessed in both preclinical animal models and cancer patients (5), but very few animal models are available for studying liver cancer immunity. The development of transgenic (tg) mice expressing tissue-specific oncogenes, such as SV40 T Ag (SV40-T), have provided important insights into the molecular events responsible for tumor pathogenesis (6). However, these models are of limited use for immunotherapeutic studies, because these mice, expressing a tissue-specific oncogene from birth and harboring tumor in the target organ, displayed a lack of immune reaction toward the tumorigenic tissue. This default of a cell-mediated immune response specific for tumor-associated Ag has been linked to various mechanisms of peripheral tolerance, including clonal deletion (7). In animals expressing SV40 T in the liver, peptide immunization does not lead to tumor regression (8). However, we have shown previously that adoptive transfer of SV40-T epitope-specific $CD8^+$ T cells induces tumor regressions in these transgenic mice (8). This indicates that the tumor cells present MHC-restricted SV40 T-derived peptides and are targets for the $CD8^+$ T cells. However, because hepatocytes are all transgenic, the inhibition is only transitory because the eliminated hepatocytes are replaced by new tg hepatocytes. The majority of liver cancer occurs relatively late after birth, and tumoral hepatocytes may express neoantigen for which neonatal tolerance does not occur. To mimic this phenomenon, new tg models have been developed in which expression of the SV40 T Ag is inducible (9). However, like the previous SV40 tg model, they suffer the same drawbacks, because all hepatocytes also express SV40-T after induction.

In this study we describe a new model where liver tumor grows progressively in fully immunocompetent mice. This model is based upon transplantation of hepatocytes from SV40-T transgenic mice by intrasplenic injection into syngeneic recipient normal mice. Using this system, where hepatocytic tumors developed inside a nontumoral parenchyma, we assessed the efficacy of curative peptide-based immunization to induce a protective $CD8^+$ T cell response.

Materials and Methods

Mice

Recipient C57BL/6J mice (7–10 wk old) were purchased from Harlan (Gannat, France). Donor tg C57BL/6J mice carry SV40-T early genes controlled by the antithrombin III liver-specific promoter on the Y chromosome (6, 8). All tg males died with liver tumors before 6 mo of age. Mice were bred in our specific pathogen-free animal facility. Experiments were performed in compliance with the French Ministry of Agriculture regulations for animal experimentation (1987).

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[4] Abbreviations used in this paper: HCC, hepatocellular carcinoma; HBVc, hepatitis B virus core protein; SFC, spot-forming cell; SV40-T, SV40 large T Ag; tg, transgenic.

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Hepatocyte transplantation

Three- to 4-mo-old SV40-T tg mice were used as donors for tumor hepatic cells. Hepatocytes were isolated by a two-step collagenase H (Roche, Mannheim, Germany) perfusion of liver fragments and were further purified over a 40% Percoll gradient (Pharmacia Biotech, Uppsala, Sweden) (10). Assessment of the purity of the hepatocyte suspension and contamination by nonparenchymal liver cells is routinely checked by cytofluorometric analysis. T cells were identified as $CD3^+$ cells, using PE-conjugated rat anti-CD3 mAb (clone 17A2; BD Pharmingen, San Diego CA). Macrophages were identified as $F4/80^+$ cells using a biotinylated rat IgG2b anti-mouse F4/80 mAb (Tebu, Le Perray-en-Yvelines, France). Endothelial cells were identified using a rat biotinylated anti-CD31 mAb (clone 390; BD Pharmingen). Streptavidin-FITC (BD Pharmingen) was used as secondary reagent. Routinely, nonparenchymal liver cells represented <30% of the total cell preparation. After Percoll purification, hepatocyte viability was at least 60%, as assessed by trypan blue dye exclusion.

Normal male C57BL/6J recipient mice were transplanted with 0.2–2 $\times$ 10^6 hepatocytes by intrasplenic injection after receiving anesthesia induced by injection of a mix of xylazine (0.2 mg/mouse; Sigma-Chemie, St. Quentin l'Arbresles, France) and ketamine (3 mg/mouse; Sigma-Chemie). To optimize experimental conditions to obtain slow-growing liver tumors, as observed for spontaneous tumors in all transplanted animals, the outcomes of transplantation with different numbers of tg hepatocytes into recipient mice were compared. In animals receiving $<4 \times 10^5$ tg hepatocytes, only a small proportion of transplanted mice (50% or less) harbored tumors, as evidenced by patent tumor nodules seen in liver sections processed 2 mo after transplantation. In a pilot experiment, the survival of mice transplanted with large amounts of tg hepatocytes (7×10^5, 1×10^6, and 2×10^6) was assessed. Death of the transplanted mice occurred between days 80 and 230, with the longest survival time corresponding to the lowest amount of transplanted cells (7×10^5 tg hepatocytes). Using this amount of tumor cells, 50% of transplanted mice died as early as 3 mo. To ensure efficient transplantation and slow-growing tumors, we choose thereafter to use a dose of 5×10^5 tg hepatocytes for transplantation of mice. In a preliminary control experiment we have injected, by the intrasplenic route, the same number of syngenic normal hepatocytes into recipient mice. Over a 6-mo period we did not observe any mortality in transplanted mice or development of tumors in the liver.

Histological studies

Liver, lung, spleen, and kidney biopsies were removed and fixed with a solution containing 85% ethanol, 10% formalin, and 5% acetic acid before inclusion in paraffin. For each animal, two to four 4-μm sections were stained with hematoxylin, eosin, and saffron. Liver sections were used to count the number of tumor nodules in 15 consecutive fields of view using a $\times$10 objective (final magnification, $\times$125). Sections were analyzed by four investigators.

Immunization procedures

Mice (normal or transplanted) were injected s.c. at the base of the tail with a pool of four SV40-T peptides (50 μg of each peptide): SV40-$T_{205–215}$ (VSAINNYAQKL), SV40-$T_{223–231}$ (CKGVNKEYL), SV40-$T_{404–411}$ (VVYDFLKC), and SV40-$T_{488–497}$ (GQGINNLDNL). In some experiments SV40-$T_{404–411}$ peptide (50 μg) was injected alone. All immunizations with SV40-T peptides were performed with 50 μg of hepatitis B virus core protein (HBV)$c_{128–140}$ Th epitope (TPPAYRPPNAPIL) (11) and emulsified in IFA. This immunization procedure was previously defined to induce highly epitope-specific $CD8^+$ T cell response with increased frequency and affinity (12). Additional studies with tumor-free mice showed that the $CD8^+$ T cell responses were long lasting (remaining constant over 1 or 2 mo) and that repeated peptide injections did not increase the efficiency of this immunization protocol (data not shown).

Nonparenchymal liver cell purification

For each mouse, the right lobe of the liver was harvested and then perfused extensively with HEPES buffer to remove all circulating cells. Biopsies were perfused with collagenase H (Roche), followed by purification on two successive Percoll gradients (Pharmacia Biotech): 60, then 35%. Viable nonparenchymal liver cells were further enumerated and assessed by ELISPOT assay. $CD8^+$ T cells were identified by cytofluorometry using a PE-conjugated rat anti-CD3 mAb and an FITC-conjugated anti-CD8 mAb (clone 53-6.7; BD Pharmingen).

ELISPOT assay

Nitrocellulose microplates (Millipore, Bedford, MA) were coated with 4 μg/ml of an anti-mouse IFN-γ rat mAb (R4-6A2; BD Pharmingen). A total of 1–5 $\times$ 10^5 spleen cells or nonparenchymal liver cells were tested in the presence of APC (RMAS-K^d, mutant lymphoma cells derived from Rauscher virus-induced murine cell line RBL-5 and defective for endogenous peptide loading of MHC class I molecules), previously incubated with CTL epitope peptide for 1 h, and 30 U/ml human rIL2. After 12 h, the plates were washed, incubated with 4 μg/ml biotinylated anti-mouse IFN-γ rat mAb (XMG1.2; BD Pharmingen) and subsequently incubated with alkaline phosphatase-extravidin. After adding chromogenic alkaline phosphatase substrate (Bio-Rad, Hercules, CA), IFN-γ spot-forming cells (SFC) were counted under a stereomicroscope and expressed as the number of spots per million tested cells.

In vivo depletion of $CD8^+$ T cells

Rat anti-mouse CD8 mAb (clone 2.43; ATCC TIB 210; American Type Culture Collection, Manassas, VA) was purified from supernatant after ammonium sulfate precipitation. $CD8^+$ T cell depletion was performed by three i.p. injections of 300 μg of rat anti-mouse CD8 mAb: 1 day before and 3 and 7 days after immunization. Purified rat IgG (Sigma-Chemie) was used as a negative control. More than 98% of blood $CD8^+$ cells were depleted by this procedure 1 wk after the end of the treatment, as verified by cytofluorometry using a rat anti-CD8 mAb (clone 53-6.7), which recognizes an epitope different from that recognized by the depleting mAb.

SV40-T PCR detection

To verify tumor regression, liver biopsies were removed and rapidly frozen in liquid nitrogen. DNA was extracted with a DNeasy tissue kit following the manufacturer's instructions (Qiagen, Hilden, Germany). The first set of amplifications was performed using β-actin primers to ascertain that all samples contained comparable amount of mouse DNA. Then, 500 ng of each sample was used as a template for PCR, using oligonucleotides specific for the SV40-T gene: SV40-T-1, GGA ATA GTC ACC ATG AAT GAG TAC AG; and SV40-T-2, GGA CAA ACC ACA ACT AGA ATG CAG TG (annealing temperature, 56°C) (9).

Statistical analysis

Differences in tumor development were analyzed using Fisher's exact test. Differences in the number of spots and tumor foci inside two or three groups of mice were analyzed for statistical significance using, respectively, the nonparametric Mann-Whitney *U* test and one-way ANOVA, followed by the Tukey multiple comparison post-test, using PRISM software (version 3.2; GraphPad, San Diego, CA), with $p < 0.05$ as the level of significance.

Results

Transplantation of SV40-T transgenic hepatocytes into normal recipient mice induced liver tumors to grow slowly without detectable immune cell infiltrates

To establish a model where hepatic tumors grow in a normal parenchyma in a fully immunocompetent recipient animal, we transplanted SV40-T tg hepatocytes into naive syngeneic mice. Hepatocytes were isolated from SV40-T tg mice and transplanted by intrasplenic injection, a route that favors colonization of the liver (13, 14). As shown in Fig. 1, injection of 5×10^5 hepatocytes from 3- to 4-mo-old tg mice (harboring fully developed liver tumors) (15) resulted in progressive tumor growth in the recipient liver. On day 7, histological examination showed tumor microfoci in a normal hepatic parenchyma (Fig. 1*A*); on day 30, numerous and large tumor foci were observed (Fig. 1*B*); and on day 100, the entire liver appeared to be invaded by tumoral hepatocytes (Fig. 1*C*). In mice harboring a massive liver tumor 3 or 4 mo after transplantation, hepatocyte-derived metastatic cells were observed in the lung (three of three), whereas none was observed in other organs such as the kidney. It should be noted that rare hepatic foci were observed in the spleen of some mice (5 of 44), probably at the injection site (Fig. 1*D*). Strikingly, no infiltrating cells were observed in tumoral livers of all transplanted animals (Fig. 1).

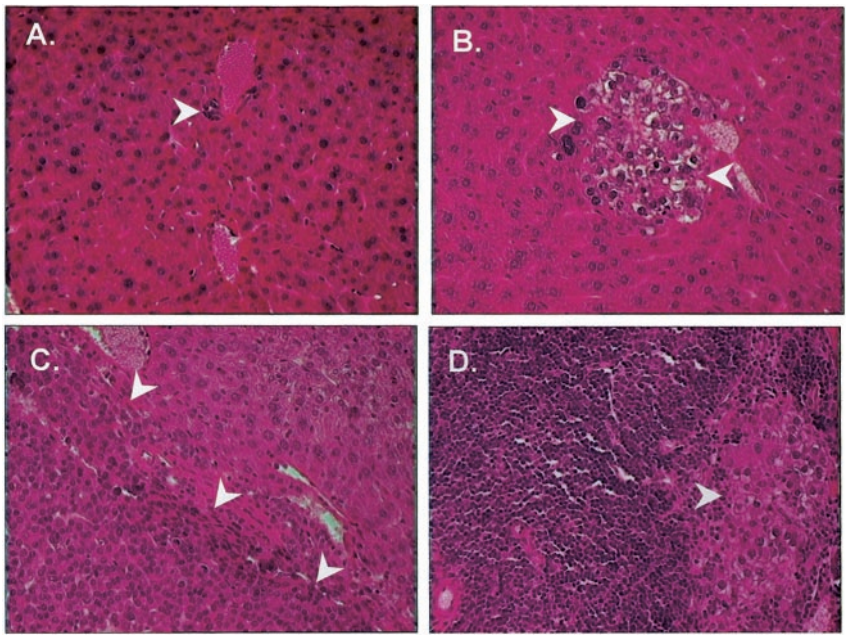

FIGURE 1. Progressive tumor growth in transplanted mice with an apparent absence of infiltrating cells. C57BL/6J mice were transplanted with 5×10^5 tg hepatocytes. Livers were analyzed 7 days (*A*), 30 days (*B*), or 100 days (*C*) after transplantation. Spleen biopsies were observed 30 days after transplantation (*D*). Results are representative of groups of five mice. Tumor nodules are shown by white arrows. Magnification, ×125.

Specific CD8$^+$ T cell response is induced by peptide immunization in tumor-bearing mice

In a previous study we have shown that SV40-T tg mice were partially tolerant to SV40-T Ags because only a weak specific CD8$^+$ T cell response was induced after immunization with SV40-T peptides (8). In these mice the expression of SV40-T Ag starts shortly after birth, and this leads to partial deletion of T cell clones with high affinity for SV40-T Ags. We thus assessed whether transplanted animals were able to mount an SV40-T-specific CD8$^+$ T cell response after peptide immunization, because this might compromise the effect of curative peptide-based immunotherapy. Transplanted mice were immunized with a pool of four SV40-T-derived peptides containing a CD8 epitope restricted by MHC class I molecules: SV40-$T_{205–215}$ (H-2K^b restricted), SV40-$T_{223–231}$ (H-2D^b restricted), and SV40-$T_{404–411}$ (H-2K^b restricted) codominant epitopes, and SV40-$T_{488–497}$ (H-2K^b restricted), a subdominant epitope (16). The peptide pool was injected s.c. together with a helper peptide from HBVc (11) that has been shown to increase the magnitude and reproducibility of CD8$^+$ T cell responses (12). Peptide immunizations were performed in mice harboring hepatic tumor initiated 1 mo earlier by injection of 5×10^5 tg hepatocytes. Because IFN-γ has been shown previously to be protective against hepatic tumor in these tg mice (10), production of this cytokine by spleen cells was studied 1 mo after immunization by ELISPOT assay. This assay was used to quantify the number of peptide-specific, IFN-γ-producing spleen cells. Responses in transplanted mice (Fig. 2*A*) were generally lower and more heterogeneous than those observed in nontransplanted mice (Fig. 2*B*). Nonetheless, a significant number of IFN-γ-producing cells was induced by the SV40-$T_{205–215}$ epitope (148 ± 69 SFC/million cells), but this number was higher with the SV40-$T_{404–411}$ peptide (200 ± 75 SFC/million cells), although this activation level was lower than that observed in immunized, but nontransplanted, C57BL/6J mice (432 ± 60 ($p = 0.0317$) and 534 ± 69 ($p = 0.0317$), respectively). The responses to peptide SV40-$T_{488–497}$ were lower, and those to SV40-$T_{223–231}$ resulted in weak IFN-γ production. These responses were maintained in tumor-bearing mice over a period of 1–2 mo (data not shown). No spontaneous response to four previously described SV40-T epitopes was detected in control, nonimmunized, transplanted mice (data not shown). Together, these results showed that, although reduced, specific T cell responses were clearly induced in transplanted mice against all peptides except SV40-$T_{223–231}$.

Immunization with SV40-$T_{404–411}$ peptide induced a specific CD8$^+$ T cell response and tumor regression

We next evaluated the effect of curative peptide immunization on tumor progression. Tumor-bearing mice were immunized 1 mo after transplantation (5×10^5 tg hepatocytes) with one of the peptides, SV40-$T_{404–411}$. This peptide was chosen because it induced the strongest response in tumor-bearing animals, and its immunogenicity was less reduced in these animals than those of the three other peptides (Fig. 2). One month after immunization, an IFN-γ ELISPOT assay was performed on spleen cells, and liver tumor growth was assessed by histology. Immunized transplanted mice had similar numbers of IFN-γ-producing cells as those measured in immunized, nontransplanted mice (Fig. 3*A*). CD8$^+$ T cells were the main producers of IFN-γ, because in vitro depletion of these cells abolished this production (data not shown). In parallel experiments, tumor-bearing mice were immunized under the same conditions with a p53 derived-peptide. However, this treatment did not induce tumor regression. Similar results were obtained in SV40-tg mice immunized with the p53-derived peptide (data not shown) (17). Transplanted mice immunized with the p53-derived peptide or control, nonimmunized, transplanted mice did not produce any IFN-γ, as assessed by ELISPOT (<10 spots/million cells), and all developed tumor (Fig. 3*B* and data not shown) with numerous (Fig. 3*C* and data not shown) and large tumor nodules (Fig. 4*A* and data not shown). By contrast, in 38% (five of 13; Fig. 3*A*, ○) of the transplanted animals immunized with the peptide SV40-$T_{404–411}$, no liver tumor foci could be detected by histology (Fig. 4*C*), and refined detection of SV40-T-persistent transgene by PCR was negative (data not shown), indicating that extensive eradication of tumor cells has occurred. Thus, only 62% (eight of 13) of the immunized transplanted mice had tumor nodules, but these were reduced in size (Fig. 4*B*) and significantly less numerous than those observed in control, nonimmunized, transplanted mice (Fig. 3*C*). In the group of mice showing only a partial therapeutic response, mononuclear and polynuclear cell infiltrates were observed

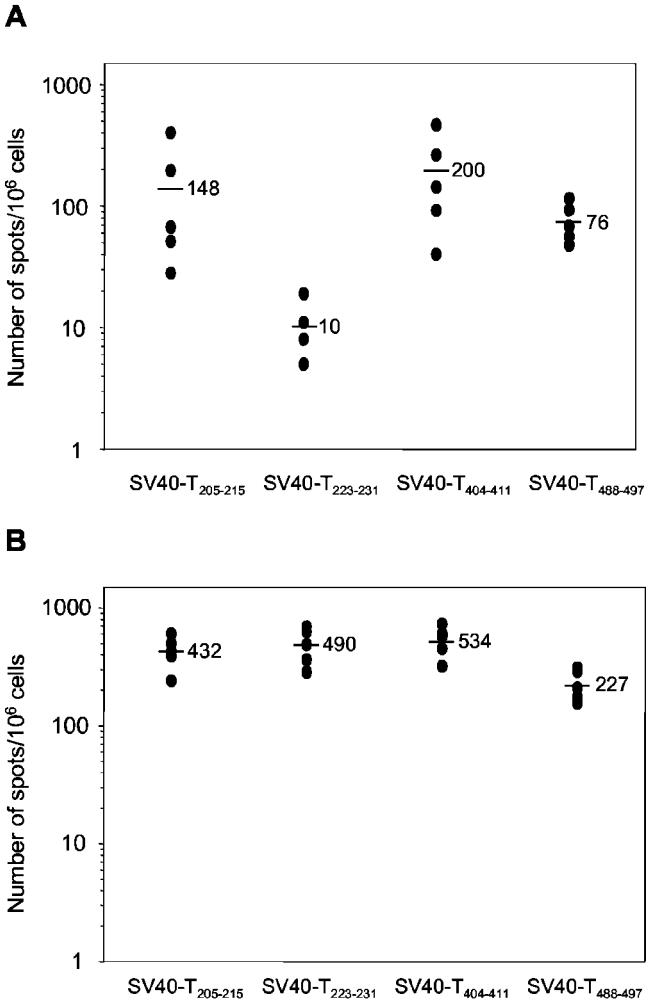

FIGURE 2. SV40-T specific $CD8^+$ T cell responses in the spleen of transplanted or nontransplanted mice after immunization. *A*, One month after transplantation with 5×10^5 tg hepatocytes, C57BL/6J mice ($n = 5$) were immunized with a pool of four SV40-T-derived peptides together with the HBVc helper peptide. *B*, Normal C57BL/6J mice ($n = 5$) were immunized in a similar way. IFN-γ production specific for each SV40-T peptide was assessed by ELISPOT with spleen cells 1 mo after immunization. Background values with no peptide or irrelevant peptide were <10 spots/10^6 cells. Data represent the average spot number in each group. As a control, transplanted or normal C57BL/6J were immunized with a p53-derived peptide together with the HBVc helper peptide. Values were <10 spots/10^6 cells.

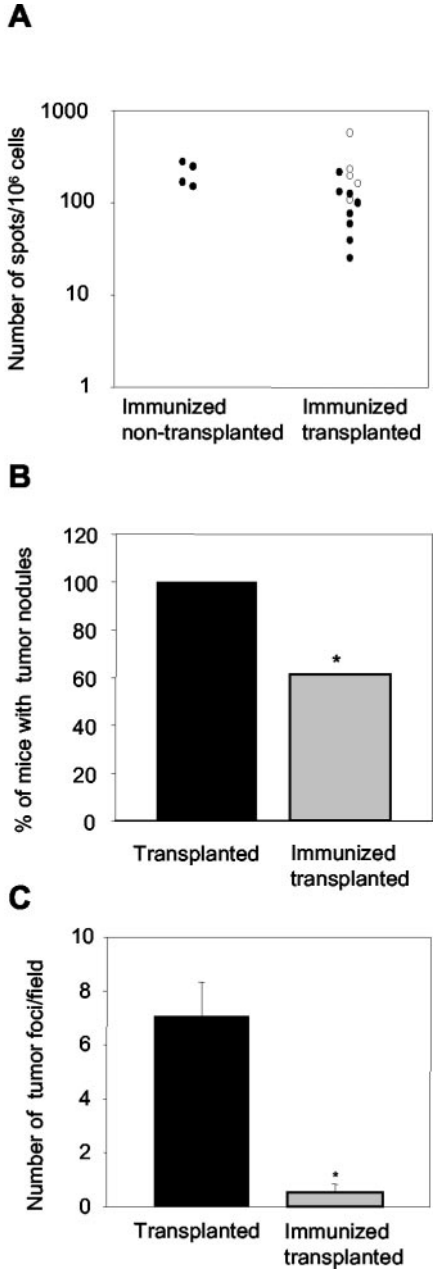

FIGURE 3. Effect of SV40-$T_{404-411}$ peptide-based immunization on liver tumor growth. One month after transplantation with 5×10^5 tg hepatocytes, C57BL/6J mice were immunized ($n = 13$) or not ($n = 5$) with SV40-$T_{404-411}$. Immunized C57BL/6J mice were used as a control ($n = 4$). *A*, IFN-γ ELISPOT assays were performed on spleen cells 1 mo after immunization. Background values with no peptide or an irrelevant peptide were <10 spots/10^6 cells. ○, Tumor-free mice as evaluated in *B*; ●, mice showing partial tumor regression. *B*, Percentage of mice with tumor nodules, as evaluated by histology. *C*, Mean tumor foci number per field using a ×10 objective (final magnification, ×125). ∗, $p < 0.05$, by Mann-Whitney *U* test.

surrounding the residual tumor nodules. To determine whether tumor could be totally eradicated, tumor regression was studied over longer time periods. A group of six mice was transplanted, and half of them were immunized with SV40-$T_{404-411}$ peptide 1 mo after transplantation. All mice were killed 3 mo after transplantation. All nonimmunized control mice developed large tumor nodules and showed no SV40-$T_{404-411}$ peptide-specific IFN-γ response, whereas the immunized mice displayed no detectable tumor, but had a high, persistent, tumor Ag-specific response (data not shown).

In addition, a group of four mice was immunized with SV40-$T_{404-411}$ peptide 2 mo after transplantation, when the tumor has invaded large areas of liver parenchyma. In these animals, tumor regression was complete (data not shown). Altogether these results indicated that a single injection of a monoepitopic peptide from tumor Ag was sufficient and as efficient as two injections to induce effective tumor regression. In a separate experiment, mice were transplanted with tumor hepatocytes, and 1 mo later they were immunized with the pool of SV40 peptides, described above, for comparison with mice immunized with the SV40-$T_{404-411}$ peptide alone. Addition of other SV40 peptides in the immunogenic formulation did not increase the number of mice without tumor or the extent of tumor regression (data not shown).

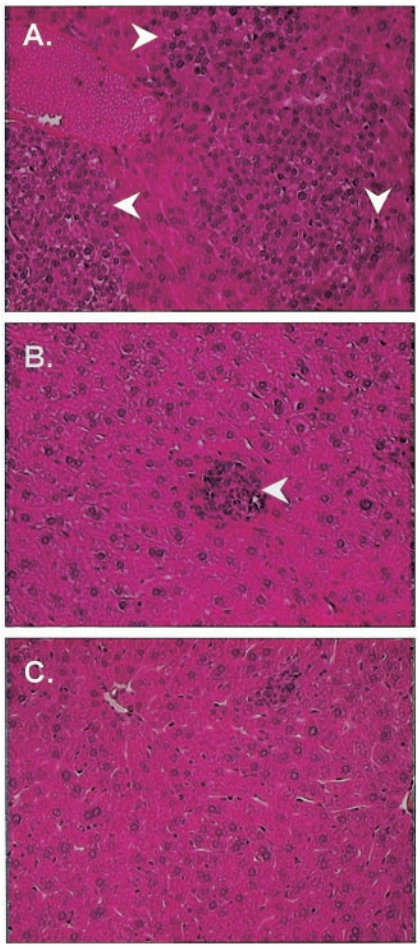

FIGURE 4. Histological evidence of the effect of the SV40-$T_{404-411}$ peptide immunization on liver tumor growth. One month after transplantation with 5×10^5 tg hepatocytes, C57BL/6J mice were immunized ($n = 13$) or not ($n = 5$) with SV40-$T_{404-411}$ peptide. Histological studies were performed 1 mo after immunization. *A*, Control, nonimmunized, transplanted mice; *B*, immunized transplanted mice with tumor nodules; *C*, immunized transplanted mice without tumor nodules. Magnification, ×125. Tumor nodules are shown by white arrows.

Active tumor regression is associated with recruitment of SV40-$T_{404-411}$ peptide-specific T cell to the tumoral liver

To quantify the $CD8^+$ T cell response at the site of tumor, we assessed the presence of SV40-$T_{404-411}$-specific T cells at the tumor site. Thus, we purified and assayed leukocytes infiltrating the tumoral livers of mice transplanted with 5×10^5 tg hepatocytes and subsequently immunized (or not) 15 days after transplantation with SV40-$T_{404-411}$. The number of $CD8^+$ T cells (detected by cytofluorometry) was not significantly different between immunized transplanted mice (192,000 ± 37,000) and nonimmunized transplanted mice (291,000 ± 47,000). As shown in Fig. 5, a high level of MHC class I-restricted T lymphocytes producing IFN-γ in response to SV40-$T_{404-411}$ was observed in the livers of immunized transplanted mice, but not in control nonimmunized transplanted mice or naive mice (data not shown).

$CD8^+$ T cell depletion in immunized mice abolished peptide immunotherapy-induced regression of established tumor

Because $CD8^+$ T cells produced IFN-γ after SV40-$T_{404-411}$ immunization and migrated to the liver, we examined whether this cell type was responsible for tumor regression. Thus, these cells were selectively depleted in immunized transplanted mice by treatment with an anti-CD8 Ab at the time of immunization, corresponding to 1 mo after transplantation. One month after immunization, the number of specific IFN-γ-producing cells in the spleen was quantified, and liver tumor growth was assessed by histology. As shown in Fig. 6, all CD8-depleted, immunized, transplanted mice had a decrease of 90% of SV40-$T_{404-411}$-specific $CD8^+$ T cells, corresponding to the efficacy of CD8 depletion (Fig. 6*A*) and developed a tumor (five of five; Fig. 6*D*). In contrast, none of the immunized transplanted mice developed liver tumor, as assessed by histology and SV40-T-specific PCR (zero of three; $p = 0.0179$ vs CD8-depleted immunized transplanted mice; Fig. 6*C*), and this regression was associated with a strong SV40-$T_{404-411}$-specific $CD8^+$ T cell response (Fig. 6*A*). All control, nonimmunized, transplanted mice developed liver tumor (three of three; Fig. 6*B*) and showed no SV40-$T_{404-411}$-specific $CD8^+$ T cell response (Fig. 6*A*). Together, these data showed that SV40-$T_{404-411}$-specific $CD8^+$ T cells activated by immunization were the immune effectors inducing liver tumor regression.

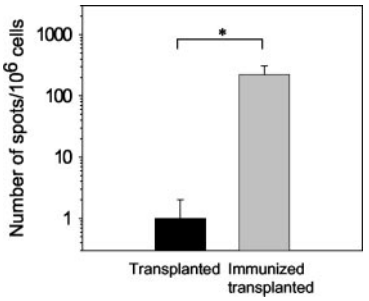

FIGURE 5. SV40-$T_{404-411}$-specific $CD8^+$ T cells producing IFN-γ in the liver of immunized transplanted mice. An IFN-γ ELISPOT assay of nonparenchymal liver cells was performed 1 mo after transplantation with 5×10^5 tg hepatocytes in mice immunized ($n = 5$) or not ($n = 5$) with SV40-$T_{404-411}$ peptide 15 days previously. Background values with no peptide or irrelevant peptide were <10 spots/10^6 cells. *, $p < 0.05$, by Mann-Whitney *U* test.

Discussion

In this study we have shown that immunization with a single injection of a specific monoepitopic peptide induced marked tumor regression in a new experimental model of liver cancer in which hepatocytic tumor cells grow in normal liver parenchyma during adulthood. Compared with the SV40-T tg mice in which all hepatocytes express SV40-T Ag (6, 8), this model more closely reflects the natural situation of human liver tumors, where only a small proportion of cells are tumoral at the start of cancerogenesis. It is also more relevant than models using transplantation of hepatoma cell lines ectopically, i.e., under the skin (18, 19). It is more practical than those using orthotopic transplantation (20) or using mice transgenic for the HBV, thymectomized, irradiated, and reconstituted with bone marrow from non-tg mice (21). In our system, intrasplenic injection of tg hepatocytes allowed their migration specifically to the liver and determined their successful engraftment in between normal parenchymal cells. Transplantation of a minimum of 5×10^5 SV40-T tg hepatocytes into syngeneic mice was necessary to reproducibly obtain the development of tumor in liver, from which mice died 3–8 mo later (data not shown). One important feature of our model is that liver tumors grow in fully immunocompetent mice. This differs from models in which xenograft tumors are inserted into immunodeficient mice (22) and allow for the evaluation of immunotherapy. Strikingly, transplantation of hepatocytes expressing SV40-T oncogene into the spleen did not induce a detectable $CD8^+$ T cell response in the spleen or an

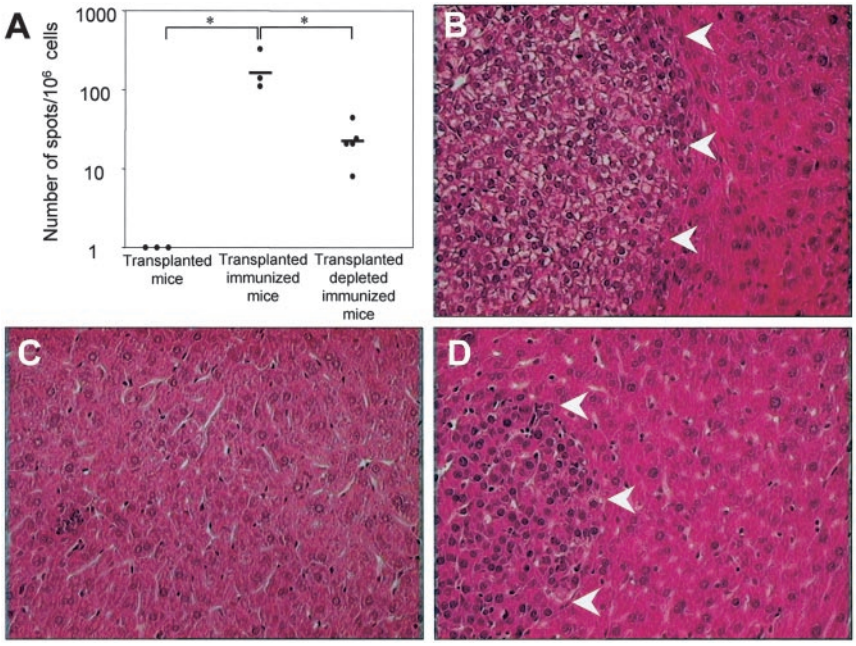

FIGURE 6. Role of CD8$^+$ T cells in tumor regression. One month after transplantation with 5×10^5 tg hepatocytes, mice were immunized with SV40-$T_{404-411}$ peptide and treated with anti-CD8 mAbs ($n = 5$; *A* and *D*) or control rat IgG ($n = 3$; *A* and *C*). Transplanted mice were used as a control of tumor growth ($n = 3$; *A* and *B*). *A*, IFN-γ ELISPOT assays performed on spleen cells 1 mo after immunization. Background values with no peptide or irrelevant peptide were <10 spots/10^6 cells. *, $p < 0.05$, by one-way ANOVA, followed by Tukey test. *B–D*, Liver histological analysis was performed 1 mo after immunization. Final magnification, $\times 125$. Tumor nodules are shown by white arrows.

infiltration of leukocytes into the liver after settlement and multiplication of the tg hepatocytes in this organ. Indeed, no detectable IFN-γ-producing CD8$^+$ T cells against the SV40-T Ag were observed, even at early stages of tumor progression (our unpublished observations). Although we did not look for CD4$^+$ T cell or Ab responses, this absence of a CD8 immune response was surprising. This clearly differs from experiments in which the intrasplenic injection of *Plasmodium*-infected hepatocytes in mice induced a strong T cell immunity against the parasite without a detectable Ab response (23). Different hypotheses can be proposed to explain this apparent absence of cellular reaction: anergy, ignorance, or peripheral tolerance of T cells. Anergy of T cells (due to absence of costimulatory molecules on tumor cells) (24) alone does not seem to be involved, because peptide immunization induced a strong specific immune response, as assessed by IFN-γ production (Figs. 2 and 3). Immunological ignorance of T cells toward tumor cells has been recently described as a default for these cells to enter secondary lymphoid organs (25). However, in our model, tg hepatocytes were injected directly into the spleen and sometimes led to small tumor foci in splenic parenchyma, probably at the injection site (Fig. 1*D*). Another explanation that has been elegantly put forward by the group of Zinkernagel (26) is the formation of a fibrous wall around tumor cells that prevents contact with T cells in lymphoid organs. No fibrous wall around tg hepatocytes was observed in the spleens of transplanted mice (data not shown); thus, this cannot explain the absence of a CD8$^+$ T response. Lastly, peripheral tolerance of T cells can occur because of immunosuppressive factors, such as TGF-β and IL-10, secreted by tumor cells (15, 27) or by the action of regulatory T cells (28). This may result in the suppression of activation or IFN-γ production by tumor-specific CD8$^+$ T cells (29). However, these tolerogenic pathways are unlikely to be involved in our model, because CD25 depletion or TGF-β neutralization by Ab treatment did not result in detection of IFN-γ-producing CD8$^+$ T cells in transplanted mice (our unpublished observations).

Despite the absence of spontaneous response to tumor cells in control, nonimmunized, transplanted mice, a strong specific CD8$^+$ T cell response was induced by immunization with a pool of SV40-T-derived peptides (Fig. 2). A hierarchy of the four epitopes was detected. Indeed, the number of peptide-specific IFN-γ-producing cells was the highest with the codominant peptide SV40-$T_{404-411}$. A lower, but significant, response was observed with the second codominant SV40-$T_{205-215}$ peptide, whereas a median response and a low response were observed, respectively, with the subdominant SV40-$T_{488-497}$ peptide and the third codominant SV40-$T_{223-231}$ peptide. It should be noted that a hierarchy in epitope-specific response alteration cannot be deduced from previous studies concerning peptide affinity to MHC molecules or sensitization of target cells (30, 31). Nevertheless these results suggest that the SV40-$T_{223-231}$-specific T cells were partially tolerated in transplanted mice. The CD8$^+$ T cell response in immunized transplanted mice was also different from that obtained with immunized SV40-T tg mice, because these mice displayed a highly impaired response to SV40-$T_{404-411}$ (8). These results also strongly suggest that the development of the tumor in recipient mice has an effect on the CD8$^+$ T cell response. CD8$^+$ T cells could still be primed by peptide immunization (presumably in draining lymph nodes), but the extent of the response was reduced compared with that in peptide-immunized, normal mice (Fig. 2). This might be due, as proposed above, to the induction of immunosuppressive factors or of regulatory T cells by the tumors or to other mechanisms.

Curative immunization with SV40-$T_{404-411}$ peptide induced complete tumor regression in ~50% (10 of 21; cumulative results from various experiments presented in this study and data not shown) of immunized transplanted mice, depending on the experiment. Moreover, immunized transplanted mice in which tumor was observed had a significantly lower number of tumor foci with overall reduced size compared with control transplanted mice, suggesting an active immune tumor regression phase (Fig. 3*C*). Delayed examination of tumor regression after immunization may indeed favor tumor regression, as suggested by data from a small group (three of three) of mice displaying complete tumor regression 2 mo after immunization (data not shown). Whereas repetitive immunizations in different models have been necessary for prolonged antitumor efficacy (32), in our system a single immunization with SV40-$T_{404-411}$ induced a long-lasting immune response, because a large number of IFN-γ-secreting CD8$^+$ T cells were observed 1–3 mo after immunization (Fig. 3*A* and data not shown). Moreover, when transplanted mice received two or three injections

at 2-wk intervals from 2–6 wk after transplantation, identical IFN-γ responses were observed, and tumor regression was not enhanced (data not shown). These SV40-$T_{404-411}$-specific $CD8^+$ T cell responses were heterogeneous. Such heterogeneity in immune response has also been observed in different human cancers (33, 34). SV40-$T_{404-411}$-specific $CD8^+$ T cells activated by immunization in secondary lymphoid organs (Figs. 3 and 6) were able to migrate to the liver, enter the parenchyma (Fig. 5), and eliminate tumoral hepatocytes, presumably through the action of IFN-γ, thus leading to tumor regression. This is in contrast to most MHC class I epitope-based immunotherapy that led to occasional clinical responses. Of interest, previous studies suggested that no correlation could be established during Ag-specific immunotherapy between tumor response and activation of immune effector cells detected in the circulation. Indeed, in most studies in humans the magnitude of the immune response was quantified in peripheral blood, which might not be the proper readout of the immune response leading to clinically significant antitumor activity. Instead, local, intratumoral detection of immune cells might be more appropriate (35). Overall, adoptive transfer of tumor-infiltrating lymphocytes remains today the most univocal therapeutic approach from an immunological perspective (5).

In summary, we have shown that curative immunization with a single peptide of tumor Ag was able to induce peripheral and tumoral liver-infiltrating, specific $CD8^+$ T cells, resulting in tumor regression in a new model of liver cancer that shares features with human liver tumor. Together, our data suggest that peptide immunotherapy may have some promise in the treatment of human liver cancer.

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