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*J Immunol* 2006; 177:255-267; doi: 10.4049/jimmunol.177.1.255
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Accumulation of CD8+ T Cells in Advanced-Stage Tumors and Delay of Disease Progression following Secondary Immunization against an Immunorecessive Epitope

Christina M. Ryan and Todd D. Schell

Self-reactive T cells that survive the process of positive and negative selection during thymocyte development represent potential effector cells against tumors that express these same self-Ags. We have previously shown that CD8+ T lymphocytes (T_{CD8}) specific for an immunorecessive epitope, designated epitope V, from the SV40 large T Ag (Tag) escape thymic deletion in line SV11 Tag-transgenic mice. In contrast, these mice are tolerant to the three most dominant Tag epitopes. The majority of the residual epitope V-specific T_{CD8} have a low avidity for the target epitope, but a prime/boost regimen can expand higher avidity clones in vivo. Whether higher avidity T_{CD8} targeting this epitope are affected by Tag-expressing tumors in the periphery or can be recruited for control of tumor progression remains unknown. In the current study, we determined the fate of naive TCR-transgenic T_{CD8} specific for Tag epitope V (TCR-V cells) following transfer into SV11 mice bearing advanced-stage choroid plexus tumors. The results indicate that TCR-V cells are rapidly triggered by the endogenous Tag and acquire effector function, but fail to accumulate within the tumors. Primary immunization enhanced TCR-V cell frequency in the periphery and promoted entry into the brain, but a subsequent booster immunization caused a dramatic accumulation of TCR-V T cells within the tumors and inhibited tumor progression. These results indicate that epitope V provides a target for CD8+ T cells against spontaneous tumors in vivo, and suggests that epitopes with similar properties can be harnessed for tumor immunotherapy.


The Tumor responsive CD8+ T lymphocytes (T_{CD8}) present in the endogenous repertoire have often been targeted for immunotherapy of antigenic tumors (1). Numerous studies have documented the in vivo capacity of T_{CD8} to recognize and eliminate tumors in experimental models (1, 2). Central to this function is the ability of T_{CD8} to recognize tumor-associated Ag epitopes displayed by cancer cells in the context of MHC class I molecules (3). Most tumor-associated Ags that have been identified in cancer patients are self-Ags, derived from nonmutated cellular proteins (4). However, generation of the T cell repertoire normally includes the elimination of potentially self-reactive thymocytes during the process of negative selection (5). Thus, the T_{CD8} population exported from the thymus is composed primarily of cells that demonstrate low avidity for thymic-expressed self-Ags or higher avidity clones that recognize tumor-associated self-Ags that are either 1) not expressed in the thymus (4), or 2) expressed at low levels (5). Consequently, the peripheral T cell repertoire has usually been purged of T_{CD8} with the most potent antitumor reactivities (6). Therefore, investigations that seek to maximize the responsiveness of tumor-specific T_{CD8} must account for the limitations imposed by the mechanisms that maintain self-tolerance.

The T_{CD8} response directed against a given Ag can target multiple epitopes, which can be characterized as immunodominant or subdominant according to the relative frequencies of T_{CD8} elicited following immunization with wild-type (wt) Ag (7). In addition, T cells targeting immunorecessive epitopes may be induced following immunization with Ags lacking each of the immunodominant and subdominant epitopes (8, 9). Expression of tumor Ags as a self-Ag has been shown to alter the immunodominance hierarchy. This is exemplified by the appearance of T_{CD8} specific for new more dominant epitopes in self-Ag knockout hosts, indicating that T_{CD8} against the most immunodominant epitopes are eliminated in the presence of self-Ag (10–12). These findings suggest that T_{CD8} specific for subdominant or immunorecessive self/tumor Ags are less susceptible to tolerogenic mechanisms, thereby permitting the persistence of both low- and high-avidity epitope-specific T cells despite the continuous presence of Ag. A few studies have reported that immunization against subdominant tumor epitopes resulted in protection from transplantable tumor challenge in the absence of self-Ag expression (13–15) or revealed that T cells specific for an immunorecessive epitope can be recruited in the context of self-Ag expression (16–18). The ability of these T_{CD8} to control the progression of spontaneous tumors, however, was not addressed.

Mice that develop spontaneous tumors due to the transgenic expression of an oncogene provide realistic models to study the recruitment of T_{CD8} against a self/tumor Ag. When expressed as a transgene under its own promoter, the SV40 large T Ag (Tag) induces choroid plexus tumors in mice (19). Line SV11 (H-2b) mice express SV40 Tag as a transgene from the viral early region promoter (19), resulting in low-level expression in the thymus and kidney, and high-level expression in the choroid plexus within the brain ventricles (20). Initial Tag expression is detectable at 14 days.
of age, leading to appearance of neoplastic cell clusters in the choroid plexus around day 35. These papillomas progress rapidly and reproducibly, ultimately obstructing the cerebral ventricles, until mice succumb to tumor burden at a mean age of 104 days (20).

In addition to its oncogenic role in transgenic mice, the Tag is also the target of a T<sub>C</sub>D<sub>B</sub> response. In C57BL/6 mice, immunization with wt Tag induces T<sub>C</sub>D<sub>B</sub> specific for three epitopes: I (residues 206–215); II/III (residues 223–231); and IV (residues 404–411), with epitope IV being the most immunodominant (21–23). T<sub>C</sub>D<sub>B</sub> specific for a fourth epitope, designated epitope V (Tag residues 489–497), are only detected upon immunization with a Tag variant, in which epitopes I, II/III, and IV have been inactivated, or with a recombinant vaccinia virus expressing epitope V as a mini-gene (9, 24). Because these T<sub>C</sub>D<sub>B</sub> recognize epitope V derived from the wt Tag, but can only be elicited upon inactivation of the other immunodominant epitopes, epitope V has been classified as immunorecessive (25). The characteristics which contribute toward the immunorecessive nature of epitope V might also permit survival of epitope V-specific T<sub>C</sub>D<sub>B</sub> in mice that develop Tag-induced tumors. This was recently investigated in line SV11 mice, where a subset of T<sub>C</sub>D<sub>B</sub> specific for Tag epitope V are spared and seed the peripheral lymphoid tissues despite Tag expression in the thymus (17). In contrast, T<sub>C</sub>D<sub>B</sub> specific for epitopes I, II/III, and IV are deleted. Despite the absence of precursors specific for these dominant epitopes, Tag epitope V maintains its immunorecessive phenotype in SV11 mice. This is evidenced by the failure to recruit epitope V-specific T<sub>C</sub>D<sub>B</sub> upon immunization with wt Tag (Ref. 26 and our unpublished observations). Thus, it was necessary to immunize specifically against epitope V to detect T cells that recognize this immunorecessive epitope. The residual response to epitope V in SV11 mice is composed primarily of lower avidity T<sub>C</sub>D<sub>B</sub> which recognize Tag epitope V peptide 1,000- to 10,000-fold less efficiently than B6-derived T cells (17). Importantly, booster immunizations expand a population of Tag V-specific clones that effectively lyse wt Tag transformed cells and have avidities similar to T cells derived from B6 mice. Thus, a smaller number of higher avidity clones specific for the Tag epitope V are present in the endogenous repertoire of SV11 mice and these T cells remain sensitive to immunization.

Due to the low frequency of the endogenous epitope V-specific T<sub>C</sub>D<sub>B</sub> in SV11 mice, the effect of persistent Tag expression on naive epitope V-specific T cells remains unknown. Therefore, the purpose of the present investigation was to examine the fate of high-avidity T<sub>C</sub>D<sub>B</sub> specific for the immunorecessive Tag V epitope upon adoptive transfer into SV11 mice bearing advanced-stage SV40 Tag-expressing tumors. We show that epitope V-specific T<sub>C</sub>D<sub>B</sub> recognize endogenous Tag and proliferate, but fail to accumulate and do not traffic to the tumor. However, immunization specifically directed toward epitope V promotes the accumulation of transferred Tag V-specific T<sub>C</sub>D<sub>B</sub> at the tumor site and enhances SV11 survival. Our results suggest that tumor Ag epitopes similar to Tag epitope V can be targeted and exploited for the treatment of spontaneous cancer.

Materials and Methods

Mice

C57BL/6 (H-<sup>2</sup>a) mice, B6.SJL-H<sup>2</sup>-2 (H-<sup>2</sup>b) mice, and UBI-GFP/BL6 mice were purchased from The Jackson Laboratory and maintained at the animal facility of the Milton S. Hershey Medical Center. SV11 mice on the C57BL/6 background express full-length SV40 Tag under the control of the endogenous SV40 promoter/enhancer (19). The SV11 line has been maintained in the animal facility of the Milton S. Hershey Medical Center by backcrossing Tag transgene-positive males with C57BL/6 females for 54 generations. SV11 transgene-positive mice were identified by PCR amplification of the transgene as previously described (26). Transgene-positive (SV11) and transgene-negative littermates (B6) were used at 85 days of age. Line 459 mice expressing the TCR<sub>E</sub> and TCR<sub>B</sub> chains specific for Tag epitope V on the C57BL/6 background were generously provided by S. S. Tevetia (Pennsylvania State University College of Medicine, Hershey, PA) and have been previously described (27). Line 459 mice were bred with B6.SJL-H<sup>2</sup>-2 mice to generate the Tag epitope V-specific TCR-transgenic (TCR-V/CD45.1 congenic mouse strain, hereafter referred to as TCR-V). UBI-GFP/BL6 mice express GFP from the human ubiquitin C promoter (28). TCR-V mice were bred with UBI-GFP/BL6 mice to generate TCR-V/UBI-GFP mice. All experimental protocols were performed in accordance with guidelines established by the institutional animal care and use committee of the Pennsylvania State University College of Medicine and complied with federal guidelines.

Cell lines and media

B6/T116A1 cells (B6/V-only Tag) express a Tag variant in which epitopes I (residues 207–215) and II/III (residues 223–231) are deleted and epitope IV is inactivated by alanine substitution of residues 406, 408, and 411, but in which epitope V remains intact (29). The cell line B6/122B1 (Tag epitope-null) expresses a Tag derivative in which all four T<sub>C</sub>D<sub>B</sub> epitopes (I, II/III, IV, and V) were inactivated by substitution of critical MHC class I anchor residues (N210A, N227A, F408A, and N493A) (29). Cell lines were maintained in DMEM, supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 100 mg/ml kanamycin, 2 mM l-glutamine, 10 mM HEPES, 0.075% (w/v) NaHCO<sub>3</sub>, and 10% FBS. Ex vivo lymphocytes were maintained in complete RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 50 μM 2-ME.

Synthetic peptides

Peptides were synthesized at the Macromolecular Core Facility of the Milton S. Hershey Medical Center by F-moc chemistry using an automated peptide synthesizer (9050 Milligen PepSynthesizer; MilliPore). Peptides were solubilized in DMSO and diluted to 5 μM in RPMI 1640 medium. Peptides used for the intracellular cytokine assay corresponded to the SV40 Tag epitope V (QGINNLDNL; peptide V) and influenza virus nucleoprotein 366–374 (ASNNEMMET; peptide Flu) (30).

Adoptive transfer and immunization protocols

For adoptive transfer, RBC-depleted lymphocytes derived from spleens and lymph nodes (LNs) of TCR-V/CD45.1-transgenic mice were resuspended in HBSS, filtered, and injected i.v. in 0.4 ml into the tail vein of B6 or B6 transgene-negative littermates at a dose of 5 × 10<sup>7</sup> clonotypic TCR-V cells/mouse. For the in vivo cytotoxicity assays, TCR-V/CD45.2<sup>+</sup> mice were used as donors to identify CD45.1<sup>+</sup> target cells. For immunization, B6/V-only Tag cells were resuspended in HBSS and injected in 0.5 ml via the i.p. route at a dose of 5 × 10<sup>7</sup> B6/V-only Tag cells/mouse. Primary immunizations were administered on the day of adoptive transfer; booster immunizations were administered on day 7 postadoptive transfer.

Lymphocyte isolation

Mice were anesthetized via i.p. injection of sodium pentobarbital (70 mg/kg body weight) diluted in 10% ethanol and perfused transcardially with PBS. Spleens, brains, cervical LN (CLN), and inguinal LN (ILN) were dissected and transferred to cold RPMI 1640. Spleens and LNs were processed to single-cell suspensions and spleens were depleted of RBCs using Tris NH<sub>4</sub>Cl. Lymphocytes were isolated from brains as described previously (31).

MHC class I tetramers and Abs

MHC class I tetramers complexes corresponding to the H-2D<sup>0</sup>/Tag epitope V (D<sup>0</sup>/V) and H-2D<sup>0</sup>/influenza virus NP epitope 366–374 (D<sup>0</sup>/Flu) conjugated with streptavidin-PE were prepared as previously described (29). Purified anti-CD16/CD32 was purchased from BD Pharmingen. The following Abs were purchased from eBioscience: PE-Cy5-labeled anti-mouse CD44 (clone IM7), FITC-labeled anti-mouse CD44 (clone IM7), FITC-labeled anti-mouse L-selectin (clone ME-14), FITC-labeled anti-mouse CD69 (clone H1.2F3), FITC-labeled anti-mouse CD45.1 (clone A20), biotinylated anti-mouse CD45.1 (clone A20), allophycocyanin-labeled anti-mouse CD44 (clone IM7), and allophycocyanin-labeled anti-mouse L-selectin (clone ME-14). Unlabeled streptavidin and streptavidin-Alexa-647 were purchased from Molecular Probes.
Flow cytometric analysis

For quantitative ex vivo characterization of isolated T cells, lymphocytes were resuspended at 2 × 10^6/ml in FACS buffer (PBS containing 2%FBS/0.01% NaN_3) and incubated in the presence of anti-CD16/CD32 and unconjugated streptavidin for 30 min on ice. Cells were washed in FACS buffer and resuspended in a mixture of the indicated fluorochrome-conjugated Ab and tetramer. PE-conjugated tetramers were diluted 1/200; FITC-conjugated mAbs (1/50); all other mAbs were diluted 1/100. Cells were stained in the dark on ice for 1 h. In the case of biotin-conjugated mAb-containing mixtures, cells were washed three times in FACS buffer and resuspended in streptavidin-conjugated Alexa 647 (Molecular Probes) at 1/500 dilution for 30 min. Cells were washed three times in FACS buffer, and fixed with 2% paraformaldehyde/PBS and analyzed using a FACScan or FACSCalibur flow cytometer (BD Biosciences). Routinely, buffer, and fixed with 2% paraformaldehyde/PBS and analyzed using a FACScan or FACSCalibur flow cytometer (BD Biosciences). Routinely, 50,000 events were recorded. Data were analyzed using FlowJo software (Tree Star).

Intracellular cytokine assay

For staining for intracellular IFN-γ, lymphocytes were isolated from the spleen, CLN, and brain, processed, and incubated in 0.2 ml of RPMI 1640/10% PBS in U-bottom 96-well plates with the indicated peptides (peptide Tag V or peptide Flu) plus 1 µg/ml brefeldin A (Sigma-Aldrich) for 6 h at 37°C, 5% CO_2. CD8^+ T cells were stained for intracellular IFN-γ using the Cytofix/Cytoperm kit (BD Pharmingen) in accordance with manufacturer’s specifications as previously described (31), and analyzed by flow cytometry. The percentage of cells that stained specifically for IFN-γ following stimulation with peptide V was determined by subtracting the percentage of CD8^+ cells which stained for IFN-γ in the presence of peptide Flu.

In vivo proliferation assay

RBC-depleted lymphocytes derived from spleens and LNs of TCR-V-transgenic mice were resuspended at 1 × 10^7/ml in PBS/0.2% BSA and labeled with 5 µM CFSE (Molecular Probes) for 10 min at 37°C. Cells were washed three times with PBS/0.2% BSA, resuspended in HBSS, filtered through a cell strainer (Falcon), and injected i.v. at a dose of 5 × 10^6 donor CD8^+ T cells per recipient mouse. Cells were harvested at different intervals and analyzed by flow cytometry for the percentage of donor CFSE^low cells.

In vivo cytotoxicity assay

In vivo cytotoxicity assays were performed as described previously (27). In brief, target cells were prepared from sex-matched B6.SJL (CD45.1^+^) spleen cells by incubation in the presence of 1 µM of the indicated peptides (peptide Tag V or peptide Flu) in RPMI 1640/10% FBS at 37°C for 90 min and then washed three times to remove excess peptide. Targets were differentially labeled with CFSE (5 µM for peptide V; 0.5 µM for peptide Flu) and then incubated for 30 min at 37°C in PBS/0.1% BSA and washed three times. A total of 5 × 10^5 target cells (2.5 × 10^4 of each) was injected i.v. into the tail vein in 0.4 ml of HBSS. The elimination of CD45.1^+^ CFSE-labeled targets was assessed the next day. The following formula was used to determine the percentage of specific killing: % lysis = (1 − (ratio unprimed/ratio primed)) × 100, where ratio = (percentage of CFSE^low donors/percentage of CFSE^high donors).

Immunohistochemistry

Brains were harvested from perfused mice as described above, imbedded in optimal temperature compound (Tissue-Tek; Sakura Finetek), snap-frozen in liquid nitrogen and placed at −80°C. Ten-micrometer sections were cut using a cryostat (Bright OFT; Hacker Instruments), mounted on slides, fixed in acetone at −20°C for 10 min, and air-dried at room temperature. Endogenous peroxidase was quenched by incubation of sections in 0.3% H2O2/PBS for 15 min at room temperature followed by washing. Sections were blocked with normal goat serum (Histomark) for 30 min and stained with primary biotinylated rat anti-mouse CD8a mAb (clone 53-6-7; BD Pharmingen) at room temperature in a humidified chamber for 1 h. Sections were washed twice in PBS and stained with biotinylated goat anti-rat IgG (H + L) secondary mAb (Histomark) for 1 h. Controls were stained with secondary mAb only. Slides were washed twice in PBS, incubated in streptavidin-peroxidase (ChemMate kit; Ventana) for 30 min and washed in PBS. The chromagen diaminobenzidine (ChemMate) was added for 2–10 min, and slides were washed in PBS followed by dH2O. Finally, sections were mounted in Crystal/Mount (Biomeda) and examined using a Nikon Microphot-FXA microscope. Representative images were captured using a Sony DCK-ST5 color digital camera.

Immunofluorescent imaging and confocal microscopy

Freshly isolated brains from perfused mice were harvested and cut coronally in half. One-half was used for flow cytometric analysis, and the other half was fixed overnight at −20°C in 4% PBS/paraformaldehyde, followed by embedding in optimal temperature compound. Brains were snap-frozen in liquid N2, cut into 10-µm sections and mounted on slides. Sections were fixed in acetone at −20°C for 10 min and air-dried at room temperature. Slides were washed two times in PBS, blocked in 10% normal goat serum for 30 min, and stained with primary mAb rat anti-mouse CD8a (clone 53-6-7) at 1/500 for 1 h in a humidified chamber at room temperature. Slides were washed three times in cold PBS and stained with secondary Alexa 647-conjugated goat anti-rat IgG (H + L) mAb (Molecular Probes) diluted 1/500 for 1 h in a humidified chamber at room temperature. Slides were washed three times in cold PBS, counterstained in 4′,6′-diamidino-2-phenylindole (DAPI) for 5 min, mounted in Aqua Poly/Mount (Poly-sciences) and coverslipped. Visualization of GFP-positive cells required no additional Ab staining but could be visualized following the postfixation step using fluorescence microscopy. Fluorescence analysis was performed with a confocal laser-scanning microscope (TCS SP2 AOBS; Leica) at 512 × 512-pixel resolution. Images were maximum projections of z-stacks. The brightness and contrast of some images were adjusted with image-analysis software (Photoshop; Adobe) and the digital resolution was held constant.

Lifespan analysis

SV11 mice were monitored for the development of hydrocephalus, indicative of end-stage choroid plexus tumors. Mice were euthanized following the development of neurological symptoms. The presence of tumors was confirmed by gross examination. In some cases, spleens and brains were processed and stained for flow cytometric detection of TCR-V cells. Survival curves were constructed by the Kaplan-Meier method with GraphPad Prism software (GraphPad Software). Significance was determined by single-factor ANOVA, and validated using the log-rank test. Values of p < 0.05 were considered significant.

Results

Naive TCR-transgenic TCD8 (TCR-V) recognize endogenous epitope V in SV11 mice

The low frequency of Tag-V-specific TCD8 in the SV11 natural repertoire has precluded direct observation of the effect of endogenous Tag expression on higher-avidity T cells specific for this epitope in tumor-bearing mice. To study the behavior of naive Tag-V-specific TCD8 in response to Tag-expressing tumors, epitope V-specific TCD8 from TCR-transgenic mice were transferred into SV11 mice with advanced tumors. Line TCR-V-transgenic mice express the TCR α- and β-chains derived from the epitope V-specific clone Y-5 (9) on the C57BL/6 background and have been previously characterized (17, 27). The epitope V-specific Y-5 clone was generated in a C57BL/6 mouse immunized with a Tag variant cell line expressing only the immunorecessive epitope V (9). This clone is thus representative of a Tag-V-specific clone from a normal T cell repertoire. TCR-V mice were bred with B6.SJL mice so that donor CD45.1^+^ TCR-V cells could be distinguished from CD45.2^+^ host cells.

Naive TCR-V lymphocytes were adoptively transferred into 85-day-old SV11 mice bearing advanced choroid plexus tumors or into Tag transgene-negative littermates, hereafter referred to as B6 mice. Some of the mice were immunized at the time of transfer with Tag-transformed B6 cells expressing a Tag variant in which the immunodominant epitopes I, II/III, and IV were inactivated (V-only Tag). Seven days posttransfer, splenocytes were analyzed for frequency and activation status of recovered TCR-V cells, detected by staining with MHC class I tetramers. Immunized B6 mice demonstrated a 20-fold increase in total tetramer-V^+^ T cells over the baseline detected in unimmunized B6 mice, 2 × 10^6 cells and 1 × 10^5 cells, respectively (Fig. 1A). Immunization of B6 mice induced an activated phenotype in TCR-V TCD8 consistent with their extensive accumulation. Nearly the entire population expressed high levels of CD44 while 75% down-regulated the LN.
Naive TCR-V T<sub>CD8</sub> recognize endogenous wt Tag in SV11 mice. A, Naive TCR-V lymphocytes (5 × 10<sup>6</sup> cells/mouse) were adoptively transferred into 85-day-old SV11 mice or Tag transgene-negative (B6) littermates. Some mice were immunized (prime) at the time of transfer with B6/V-only Tag cells (5 × 10<sup>5</sup> cells). Seven days posttransfer, splenocytes were analyzed for frequency of CD8<sup>+</sup>/tetramer<sup>+</sup>-V<sub>T</sub> cells and indicated as percent of total CD8<sup>+</sup> cells and total number of epitope-specific splenocytes (left panels). Total numbers represent the mean of three mice per group and the experiment was performed three times. Recovered cells were costained for donor origin (CD45.1), and activation status (CD44 and L-selectin). The percent of CD45.1<sup>+</sup>, CD44<sup>hi</sup>, and L-selectin<sup>lo</sup> cells is indicated. B, TCR-V cells were labeled with CFSE before adoptive transfer of 5 × 10<sup>5</sup> cells into 85-day-old SV11 mice or B6 littermates. At 7 days posttransfer, CD8<sup>+</sup>/Tet<sup>+</sup>-V<sub>T</sub> cells from the spleen and CLN were examined for proliferative status. The percent of CD8<sup>+</sup>/Tet<sup>+</sup>-V<sub>T</sub> cells that remain undivided (right marker), had divided one to six times (center marker), or greater than six times (left marker) is indicated.

**Differential kinetics of TCR-V T cell accumulation in response to endogenous Tag vs immunization**

There are several potential explanations for the failure of TCR-V cells to accumulate in the spleens and CLN of naive SV11 mice by 7 days postadoptive transfer. These include: 1) an earlier peak in expansion; 2) migration to a different anatomic site following activation; or 3) deletion. To examine whether the peak of the proliferative response preceded our day 7 analysis time point, we performed a kinetic study to monitor the frequency, activation status, and proliferation of TCR-V cells in the spleen, draining CLN, and nondraining ILN at specified time points following TCR-V cell transfer. The frequency of tetramer<sup>-</sup>/CD8<sup>+</sup> T cells recovered from the spleen, CLN, and ILN was assessed at 24, 48, and 96 h as well as 7 and 14 days post-TCR-V cell transfer.

In immunized B6 mice, the peak of the response in the spleen occurred on day 7, with TCR-V cells comprising 1.8 × 10<sup>6</sup> cells/spleen (Fig. 2A). By contrast, TCR-V cell frequency in the spleen of naive SV11 mice peaked at day 4, comprising an average of 5 × 10<sup>5</sup> cells/spleen. Although the frequency of TCR-V T cells in the spleen at day 4 postadoptive transfer was similar in both naive and immunized SV11 mice, immunization delayed the peak of TCR-V T cell accumulation until day 7. The kinetics of TCR-V cell accumulation in the CLN of B6 mice was similar to that observed in the spleen, peaking 7 days postimmunization (Fig. 2A). In contrast, the frequency of TCR-V cells increased dramatically in the CLN of naive SV11 mice. Unlike in the spleen, immunization only minimally increased the percentage of TCR-V cells that had undergone seven or more divisions in the CLN. These results indicate that recognition of endogenous Tag in naive SV11 mice leads to a modest degree of expansion consistent with the presence of proliferating cells. Immunization doubles the overall expansion of TCR-V cells detectable in the spleen and lymphoid organs of SV11 mice. However, while the proportion of dividing cells in immunized B6 and SV11 mice is the same, fewer cells accumulate in the spleens of SV11 mice.
FIGURE 2. Kinetics of TCR-V T cell activation and accumulation in response to endogenous Tag vs immunization. A and B, Naive TCR-V cells were transferred into groups of SV11 or B6 hosts (three mice per group). Some mice in each group also received B6/V-only T Ag immunization. A, The total number of CD8\(^+\)/tetramer-V\(^+\) cells recovered from the spleen, CLN, and ILN was assessed at 24, 48, and 96 h as well as 7 and 14 days posttransfer. B, The expression of activation markers CD69, and CD44 was assessed on gated populations of CD8\(^+\)/Tet-V\(^+\)/CD45.1\(^+\) cells recovered from the spleen and CLN at 2, 6, 24, 48, 72, and 96 h posttransfer of TCR-V cells. C, Naive TCR-V cells were CFSE-labeled before adoptive transfer into groups of 85-day-old SV11 or B6 mice (four mice per group). Some SV11 or B6 mice also received immunization with B6/V-only Tag cells on the day of transfer. Proliferation of CD8\(^+\)/Tet-V\(^+\)/CD45.1\(^+\) recovered cells was assessed in the spleen and CLN at 2 and 4 days posttransfer.
within the first 48 h postadoptive transfer into both naive and immunized SV11 mice. The magnitude of the response in B6 mice never reached the peak levels observed in the CLN of SV11 mice. This rapid accumulation of TCR-V cells in the CLN of SV11 mice is likely due to the presence of endogenous Tag draining from the choroid plexus. The kinetics of T cell expansion and contraction in the CLN of SV11 mice was similar regardless of whether the mice were immunized. As a control for the tumor-draining CLN, a third anatomic site—nondraining ILN—was also analyzed. The kinetics of TCR-V cell accumulation in the ILN paralleled the results measured in the spleen (data not shown). These results reveal that 1) the kinetics of TCR-V accumulation in the spleen is similar in SV11 and B6 mice following immunization, despite less extensive expansion in SV11 mice, 2) immunization of SV11 mice delays the peak of TCR-V accumulation in the spleen, and 3) TCR-V cells accumulate rapidly in the CLN in response to the endogenous Tag.

Endogenous Tag triggers rapid activation of naive TCR-V cells in SV11 mice

The accelerated accumulation of TCR-V cells in the tumor-draining CLN of SV11 mice suggests that TCR-V cells encounter endogenous Tag earlier than Tag derived from cellular immunization. To address this question, we compared the initial response of TCR-V cells to endogenous Tag in naive SV11 mice with the response to immunization in B6 mice. The expression of activation markers CD69 and CD44 was assessed on gated populations of CD8<sup>+</sup> Tet-V<sup>+</sup>CD45.1<sup>+</sup> cells at 2, 6, 24, 48, 72, and 96 h post-transfer of TCR-V cells. CD69 was initially detected on TCR-V T cells recovered from the spleens of B6 mice between 24 and 48 h after adoptive transfer and immunization (Fig. 2B, left panels). In the CLN, up-regulation of CD69 was not observed until 72 h. A more rapid kinetic profile was observed in naive SV11 mice. Expression of CD69 on TCR-V T cells was first detected at 6 h on cells from the spleen and 2 h on cells from the CLN. In addition, the percentage of TCR-V T cells that up-regulated CD69 after transfer into SV11 mice was significantly higher than in immunized B6 mice. This might be explained by a larger proportion of TCR-V T cells being initially activated by the endogenous Tag than by immunization. Alternatively, because CD69 expression is transient, TCR-V T cells activated by immunization might more rapidly down-regulate CD69 expression than T cells activated by the endogenous Tag. Thus, expression of CD44, an activation marker that retains stable high-level expression on the surface of Ag-experienced cells, was also examined. Before adoptive transfer, ~12% of TCR-V cells were CD44<sup>high</sup> and upon transfer to naive B6 mice, 20–30% of TCR-V cells recovered from the spleen and CLN were CD44<sup>high</sup>. This increase is not likely due to homoeostatic proliferation of transferred TCR-V cells because they fail to proliferate in B6 hosts (Fig. 1B), but might be due to preferential retention of CD44<sup>high</sup> cells in the lymphoid organs of naive mice. Following immunization of B6 mice, 75 and 50% of TCR-V cells in the spleen and CLN, respectively, were CD44<sup>high</sup> by 72 h. By 96 h postimmunization, 90% of the TCR-V cells in both organs were CD44<sup>high</sup>. In contrast, upon transfer to naive SV11 mice, 75% of the TCR-V cells in the spleen were CD44<sup>high</sup> by 48 h, 24 h earlier than what was observed in immunized B6 mice. A similar profile was observed in the CLN, where 80% of TCR-V cells were CD44<sup>high</sup> by 48 h. Thus, the kinetics of CD44 expression suggest that TCR-V T cells are activated rapidly against the endogenous Tag in SV11 mice with the effects of exogenous immunization delayed for 24–48 h.

Given that TCR-V T cells are phenotypically activated very early posttransfer into SV11 mice (Fig. 2B), it was plausible that they also undergo an earlier proliferative burst in the tumor-bearing environment. To address this question, proliferation of CFSE-labeled TCR-V cells was assessed at 2 and 4 days following adoptive transfer. TCR-V cells transferred to unimmunized B6 mice remained undivided at both time points (Fig. 2C). Proliferating TCR-V T cells could be detected in immunized B6 mice by day 4, but not day 2, following immunization. In contrast, TCR-V cells transferred to naive SV11 mice were actively dividing by day 2 in both the spleen and CLN, although a larger proportion of cells in the CLN had divided. The effects of immunization of SV11 mice were not realized on day 2, but by day 4, immunized SV11 mice had an increased proportion of cells that had divided in both the spleen and CLN compared with naive SV11 mice. This result is consistent with the accumulation of higher numbers of TCR-V T cells in the lymphoid organs of immunized vs naive SV11 mice.

Taken together, these results indicate that the earlier peak in TCR-V T cell accumulation in naive SV11 mice vs immunized B6 mice is due to the 24–48 h head start in TCR-V T cell activation and proliferation. The finding that subsequent immunization of SV11 mice confers similar kinetics of T cell expansion and contraction as found in B6 mice might be explained by increased triggering of naive T cells or by enhanced survival of activated cells (33). However, despite comparable kinetics, TCR-V T cell frequency in immunized SV11 mice never reached the levels observed in B6 mice, suggesting that cells exposed to endogenous Tag in SV11 mice are less responsive to immunization administered on the day of adoptive transfer.

Naive TCR-V cells acquire effector function in response to endogenous Tag in SV11 mice

To determine whether TCR-V T cells responding to the endogenous Tag acquire effector function in tumor-bearing SV11 mice, we assessed both IFN-γ production by donor TCR-V T cells and in vivo killing of epitope V-pulsed target cells in tumor-bearing mice. Naive TCR-V cells were transferred into SV11 or B6 mice at 85 days of age. Some mice were immunized with B6/V-only Tag cells on the day of adoptive transfer. Seven days later, spleens were harvested and the frequency of peptide-specific IFN-γ-producing cells was determined. The number of CD8<sup>+</sup> splenocytes producing IFN-γ in response to epitope V peptide was directly compared with the number of tetramer-V<sup>+</sup>CD8<sup>+</sup> splenocytes to estimate the proportion of TCR-V cells harboring effector function (Fig. 3A). In immunized B6 mice, 2.1 × 10<sup>6</sup> cells were tetramer-V<sup>+</sup> and 1.6 × 10<sup>6</sup> cells secreted IFN-γ, indicating that the majority of tetramer-V<sup>+</sup> cells were functional. In naive B6 mice, the frequency of CD8<sup>+</sup> cells that produced IFN-γ in response to Tag-V peptide was at background levels (equivalent to control peptide). In naive SV11 mice, 4 × 10<sup>6</sup> splenocytes were tetramer-V<sup>+</sup> and approximately half this number of cells produced IFN-γ, demonstrating that TCR-V T cells acquired effector function following activation in naive SV11 mice. Upon immunization of SV11 mice, the number of tetramer-V<sup>+</sup> cells doubled to 8 × 10<sup>6</sup> cells and ~75% of this number produced IFN-γ in response to Tag epitope-V. These data suggest that upon immunization, the fraction of TCR-V cells with effector function is similar to that achieved in B6 mice, although the magnitude of the response is not as high.

Assays were also performed to determine the in vivo cytotoxic activity of TCR-V cells (Fig. 3B). TCR-V cells were transferred to SV11 or B6 mice with or without immunization. Seven days later, equal numbers of differentially labeled peptide-V and control peptide pulsed target cells were injected i.v. and assessed for elimination in the spleen after 12 h. Upon transfer into unimmunized B6 mice, no specific loss of either population was detected (Fig. 3B). In immunized B6 mice, virtually all peptide-V pulsed targets were...
eliminated, indicating Tag-V specific function by TCR-V cells in vivo. In unimmunized SV11 mice, 47% of epitope-V pulsed targets were eliminated, and this percentage was further increased to 68% in immunized SV11 mice. Target cells were not eliminated from SV11 mice that did not receive TCR-V T cells (data not shown). Thus, TCR-V cells are capable of developing effector function in response to endogenous Tag in tumor-bearing SV11 mice, even in the absence of immunization.

Tag epitope V-targeted immunization promotes TCR-V cell entry into the brain

The discrepancy in the total number of TCR-V T cells that accumulate in the spleens of SV11 vs B6 mice following immunization might be explained by deletion of T cells responding to endogenous tumor Ag (34–36) or, alternatively, their migration to the tumor site (37–39). To assess the potential for naive TCR-V cells to infiltrate choroid plexus tumors, TCR-V cells were transferred into SV11 or B6 mice with and without immunization and their presence in spleens and brains was assessed 7 days later. Frequencies of TCR-V cells in the spleen represented 3.8 and 2.3% of CD8+ cells in naive SV11 and B6 mice, respectively (Fig. 4). No TCR-V cells infiltrated the brains of either SV11 or B6 mice without immunization, indicating that priming of TCR-V T cells against the endogenous Tag fails to result in significant accumulation of T cells in the brains of tumor-bearing SV11 mice. Upon primary immunization, TCR-V cell frequency increased to 6.4 and 22% of CD8+ splenocytes in SV11 and B6 mice, respectively. In addition, immunization resulted in a significant influx of TCR-V T cells into the brains of both groups, representing 5.5 and 4.7% of CD8+ T cells in SV11 and B6 mice, respectively (Fig. 4). The presence of TCR-V cells in the brains of both strains of mice at this time was most likely the result of the acute response to immunization, as similar proportions of T cells reactive toward the immunodominant Tag epitope IV were previously observed to enter the brains of B6 mice following immunization (31). In the present study, we noted that similar numbers of TCR-V T cells infiltrated the brains of SV11 and B6 mice despite 3-fold lower numbers of TCR-V T cells in the spleens of SV11 mice, suggesting some
preferential accumulation in SV11 brains. Thus, specific immunization, but not endogenous Tag, promoted TCR-V cell access to the brain regardless of whether Tag was expressed in the choroid plexus or tumor was present.

A prime-boost approach promotes TCR-V cell accumulation in the brains of SV11 mice

Previous work demonstrated that the residual epitope V specific T<sub>CD8<sup>+</sub></sup> in SV11 mice are optimally expanded following a prime and boost regimen (17). Given that primary immunization promoted TCR-V cell expansion in the spleen and promoted migration to the brain (Fig. 4), we also assessed the persistence of TCR-V cells at later time points, and examined whether accumulation could be further amplified following a booster immunization. TCR-V cells were transferred into SV11 or B6 mice ± primary immunization. Seven days later, spleens and brains from representative mice were analyzed for the presence of CD8<sup>+</sup>Tet-V<sup>+</sup> cells, while some of the primed mice from each group were boosted with B6/V-only Tag immunization. All remaining mice were analyzed 20 days following the initial TCR-V cell transfer, at 103 days of age (Table I). The frequencies of TCR-V cells detected at day 7 following adoptive transfer ± immunization were similar to the data presented in Fig. 4 (Table I). By 20 days posttransfer, TCR-V cells were undetectable in the spleens and brains of both naive SV11 and B6 mice. In mice that received primary immunization, 1.2% of SV11 CD8<sup>+</sup> splenocytes and 10% of B6 CD8<sup>+</sup> splenocytes were Tet-V<sup>+</sup> at 20 days posttransfer. This represents a 4- and 2-fold respective decrease in the cell frequencies observed in SV11 and B6 mice at day 7. In the brains of primed B6 mice, 3% of CD8<sup>+</sup> cells were Tet-V<sup>+</sup>, whereas as 20% of CD8<sup>+</sup> cells were Tet-V<sup>+</sup> in SV11 brains. These values represent a 2-fold decrease for B6 mice but a 3-fold increase for SV11 mice. Thus, TCR-V T cells continue to accumulate at the tumor site between days 7 and 20 following primary immunization.

We next assessed the effect of boosting on expansion of TCR-V T cells in SV11 mice (Table I). In the spleens of primed/boosted B6 mice, TCR-V cells expanded to 25% of CD8<sup>+</sup> cells, a 1.3-fold increase over the level detected 7 days after the primary immunization. In the brains of these same mice, 7% of CD8<sup>+</sup> cells were specific for epitope V, representing only a 1.1-fold increase in the level detected at day 7. The frequency of CD8<sup>+</sup> T cells that were Tet-V<sup>+</sup> in SV11 mice following the prime/boost regimen was 5 and 76% in the spleen and brain, respectively. This represents a 1.6-fold decrease in the spleen, but a 10.5-fold increase at the tumor site in the brain. This dramatic increase in TCR-V accumulation also is reflected in that TCR-V T cells represent 22% of total brain-infiltrating lymphocytes in SV11 mice that received both a prime and boost. These data indicate that TCR-V T cells preferentially accumulate at the tumor site in SV11 mice.

Brain-infiltrating TCR-V cells detected in SV11 mice are activated and functional

Several reports in both mouse and human studies have indicated that CD8<sup>+</sup> T cells recovered from tumors are anergic (40, 41). Thus, we assessed the activation state and functional potential of TCR-V cells recovered from SV11 mice that had been primed and boosted. In the spleens of both B6 and SV11 mice, a homogenous population expressed CD44, indicating that all cells were Ag experienced (Fig. 5A). Additionally, in both of these groups the majority of cells had down-regulated L-selectin (L-selectin<sup>low</sup>), although splenocytes from SV11 mice also contained a population of L-selectin<sup>high</sup> cells. TCR-V cells recovered from the brains of both B6 and SV11 mice were homogeneously CD44<sup>high</sup> and L-selectin<sup>low</sup>, indicating that only highly activated cells were present in the brain.

To determine whether these phenotypically activated cells retained effector cytokine function, brain-infiltrating TCR-V cells in 103-day-old B6 and SV11 mice that had received adoptive transfer/prime/boost were assessed for ex vivo production of IFN-γ in response to Tag-V peptide stimulation. The percentage of Tet-V<sup>+</sup> cells detected in the spleens of B6 and SV11 mice at this time was 25 and 5% of CD8<sup>+</sup> cells, respectively (Fig. 5B). A proportion of these cells made IFN-γ, with 15 and 1.8% producing Tag-V peptide stimulated cytokine in B6 and SV11 mice, respectively. In the brain, 13% of CD8<sup>+</sup> cells in B6 mice and 76% of CD8<sup>+</sup> cells in SV11 mice were tetramer-V<sup>+</sup>. A population of brain-derived lymphocytes from both B6 (4% of CD8<sup>+</sup> cells) and SV11 (27% of CD8<sup>+</sup> cells) mice also produced IFN-γ in response to epitope V peptide (Fig. 5B), indicating that at least a subset of TCR-V cells from the tumor site of SV11 mice maintained the capacity to secrete cytokine.

Table I. Accumulation of TCR-V T cells in the brain of SV11 mice following prime/boost<sup>a</sup>

<table>
<thead>
<tr>
<th>Strain</th>
<th>Prime</th>
<th>Boost</th>
<th>Analysis</th>
<th>% of Total cells</th>
<th>% of CD8&lt;sup&gt;+&lt;/sup&gt; cells</th>
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<td></td>
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<td>(Spleen)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>Day 7</td>
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<td>0.8</td>
<td>22.0</td>
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</tbody>
</table>

<sup>a</sup> TCR-V cells were transferred into 85-day-old SV11 or B6 mice ± primary immunization with B6/V-only Tag cells. After 7 days, lymphocytes recovered from spleens and brains of representative mice were analyzed for the presence of CD8<sup>+</sup>/Tet-V<sup>+</sup> cells, while half of the remaining primed mice from each group were boosted with V-only Tag immunization. All remaining mice were analyzed on day 20 following TCR-V cell transfer, at 103 days of age. TCR-V cell frequencies in the spleen and brain are represented as percent of CD8<sup>+</sup> cells and percent of total cells. Data shown is the mean of three separate experiments, which included three mice per group.

<sup>b</sup> Values in bold highlight the increased number of cells detected in the brains of SV11 mice vs B6 mice following prime and boost.
TCR-V T cells infiltrate choroid plexus tumor stroma following prime/boost

To determine whether TCR-V T cells detected among brain-infiltrating lymphocytes could migrate into the tumor stroma in SV11 mice, we performed immunohistochemical analysis. Frozen sections were prepared from SV11 and B6 brains following 1) no treatment, 2) TCR-V cell transfer + primary immunization, and 3) TCR-V cell transfer + prime/boost. Brains were harvested at day 20 following TCR-V cell adoptive transfer and sections stained for CD8. In SV11 mice receiving no treatment, brain sections were devoid of CD8 staining (Fig. 6A). CD8⁺ cells were found within the tumor tissue of SV11 mice that received primary immunization (Fig. 6B), but the density of CD8⁺ cell infiltration increased following the booster immunization (Fig. 6C). Cells staining positive for CD8 were localized only to the tumor stroma and were not detected throughout the brain parenchyma in SV11 mice. Staining was not detected in the absence of primary anti-CD8 Ab (data not shown). Although B6 mice lack tumor mass in the choroid plexus, some CD8⁺ cells were detected scattered throughout the brain parenchyma in mice that had received TCR-V cell transfer/prime/boost (Fig. 6D), but these cells did not localize to the choroid plexus. Thus, histological analysis of SV11 brains supports the quantitative flow cytometric analysis of brain-infiltrating CD8⁺ cells in SV11 and B6 mice, indicating that CD8⁺ cells specifically infiltrate the SV11 tumor stroma following TCR-V cell transfer combined with prime/boost.

To confirm that brain-infiltrating CD8⁺ cells were actually TCR-V cells, we adoptively transferred donor lymphocytes from TCR-V-transgenic mice that had been crossed with GFP⁺ mice, such that all donor TCR-V cells were GFP⁺. Following the same immunotherapeutic regimen of TCR-V cell transfer/prime/boost, spleens and brains were harvested from SV11 and B6 mice at 103 days of age. Upon microscopic examination, GFP⁺ cells were abundant in SV11 brain sections and similar to the distribution of CD8⁺ cells in the immunohistochemical analysis, these cells appeared to cluster at specific zones in the tumor tissue (Fig. 6E). Costaining of SV11 sections with anti-CD8 revealed the specific accumulation of GFP⁺ CD8⁺ cells within the tumor and the virtual absence of these cells from the rest of the brain parenchyma visualized with DAPI (Fig. 6F). Some GFP⁺ cells appeared to be either weakly positive or negative for CD8 expression. To verify the CD8⁺ phenotype of the GFP⁺ cells present in the tumor stroma, parallel flow cytometric analysis was used to determine the proportion of GFP⁺ cells that were specific for epitope V. This analysis confirmed that 97% of GFP⁺ cells isolated from the brain were also CD8⁺ Tet-V⁺ (Fig. 6G). Few GFP⁺ CD8⁺ cells were detected in sections from B6 mice (data not shown). These data clearly indicate that TCR-V T cells specifically infiltrate choroid plexus tumors following prime/boost immunization.

Primaging and boosting promotes increased survival of tumor-bearing SV11 mice

Because TCR-V cells penetrated the brain and accumulated at the tumor in SV11 mice that received a prime/boost regimen combined with adoptive transfer, the effect of these cells on tumor growth was investigated (Fig. 7). The life spans of SV11 mice treated with the following therapeutic treatments were compared: 1) TCR-V cells only; 2) TCR-V cells + primary immunization; and 3) TCR-V cells + prime/boost. Additional control groups included 4) mice receiving no treatment; 5) mice receiving prime + boost only (no TCR-V cells); and 6) mice given TCR-V cells and primed and boosted with a cell line expressing Tag in which epitope V has been inactivated (Tag epitope-null). Adoptive transfer of TCR-V cells alone had no effect on survival of SV11 mice (Fig. 7). In addition, primary immunization of SV11 mice failed to result in an increase in the lifespan of SV11 mice, despite the infiltration of TCR-V T cells to the tumor site (see Figs. 4 and 6). Only after the TCR-V cell adoptive transfer was combined with prime/boost immunization with B6/Tag-V only cells was SV11 survival significantly enhanced. This result indicates that high levels of TCR-V T cell accumulation within the advanced-stage choroid plexus tumors is associated with a significant (p value <0.0001) increase in survival of SV11 mice.

Discussion

Vaccination approaches that target endogenous self/tumor-reactive TCD8 have met with varying levels of success in a number of tumor models (34, 42–49). Unfortunately, such self-reactive cells are often subject to peripheral tolerance, limiting their effectiveness against progressing tumors. Additionally, the functional capacity of T cell populations targeting self-Ags is often constrained by their intrinsic low avidity (11, 49), precluding recognition of natural levels of Ag expressed by tumor cells, or resulting in partial delivery of signals downstream of the TCR (50). Thus, limited by the combined effects of peripheral tolerance and/or low avidity, tumor epitope-specific TCD8 have frequently failed to exert control over established tumors (34, 41, 51).
Aware of these limitations, we focused our analysis of T<sub>CD8</sub>-targeted immunotherapy on a known immunorecessive epitope in mice bearing advanced-stage tumors. Importantly, naive TCR-V cells were found to recognize epitope V derived from the endogenous wt Tag in tumor-bearing SV11 mice, demonstrating that this epitope is expressed at significant levels in vivo despite its limited half-life with H2-D<sup>q</sup> (24). Rapid activation of TCR-V cells induced proliferation, but only low-level accumulation of T cells in the lymphoid organs was observed. Subsequent immunization expanded naive TCR-V cells and promoted their initial entry into the brain, although a booster immunization was required to achieve high-level accumulation of functional TCR-V cells in the brains and infiltration into the choroid plexus tumors. Thus, this study reveals that T<sub>CD8</sub> specific for epitope V infiltrate advanced-stage tumors, where the accumulation of T cells in the tumor stroma was associated with a delay in tumor progression.

Investigations that have explored T<sub>CD8</sub> responses to similar tumor-associated subdominant or immunorecessive epitopes expressed by spontaneous tumors. Previously, our laboratory found that Tag epitope V-specific T<sub>CD8</sub> could be expanded from the endogenous T cell repertoire in line 501 SV40 Tag-transgenic mice (18) and line SV11 mice (17). However, the effect on tumor growth was not addressed. Likewise, using the TRAMP mouse model of prostate cancer, Grossmann et al. (16) reported the ability to recruit T<sub>CD8</sub> against the SV40 Tag-V epitope, but did not assess the effect on tumor progression. Singh et al. (54) recently described the discovery of subdominant T<sub>CD8</sub> epitopes within the HER-2/neu Ag that could induce an antitumor response in mice that express HER-2/neu, but the ability of immunization to control spontaneous mammary tumors in these mice remains to be determined. Thus, to our knowledge, the effect of T<sub>CD8</sub> specific for an immunorecessive tumor epitope on spontaneous tumor progression has not previously been explored.

For an epitope to be effectively targeted in vivo, it must be efficiently presented for recognition by the responding T<sub>CD8</sub>. This is particularly important for epitopes that might be limited by Ag processing or presentation. Two lines of evidence indicate that epitope V...
is presented in vivo from wt Tag in SV11 mice. First, we found that naive TCR-V T cells are activated and proliferate in the lymphoid organs following transfer into SV11 mice, although expansion was limited. This finding is consistent with recently published data in which TCR-V cells transferred into B6 hosts are activated, but undergo minimal expansion upon immunization with wt Tag-transformed cells. The findings from Otahal et al. (27) illuminated some of the mechanisms which contribute to the immunorepressive phenotype of Tag epitope V. The weak response in B6 mice was attributed to inefficient cross-presentation of Tag-V epitope, leading to poor priming of naive epitope V-specific T cells (27). This is likely due to the formation of unstable H-2Db/epitope V complexes (24), which may fail to provide a prolonged stimulus (55). Additionally, the presence of T cells specific for immunodominant epitopes that compete for access to the same APCs may contribute toward the immunorepressive nature of Tag epitope-V specific TCDS (27). In SV11 mice, TCDS precursors specific for the three most immunodominant epitopes are absent from the residual repertoire (26). Therefore, the limited accumulation of TCR-V cells following exposure to endogenous Tag might partially be due to poor cross-presentation of the epitope. Rapid dissociation of epitope V/Dpb complexes from the cell surface may result in premature disruption of Ag engagement, preventing efficient T cell expansion (55). Whether epitope V/Dpb complexes on APC are limiting in SV11 mice remains to be determined.

The second finding indicating that epitope V is presented in vivo in SV11 mice is that TCR-V T cells accumulate specifically in the brains and choroid plexus tumors following the prime and boost immunization. Other models have also observed tumor Ag-specific accumulation of activated T cells at the tumor site (37–39, 56). These studies were primarily performed using transplantable tumors that expressed foreign Ag. By contrast, it has been reported that under tolerizing conditions, immunodominant self-epitope specific TCDS do not readily penetrate transplanted tumors that express self-Ag (34, 47). Our results show that in the context of self-Ag expression, functionally competent Tag-V-specific T cells accumulate within the tumor, indicative of specific recognition of this epitope in vivo.

A central question addressed in this study is whether high-avidity TCDS responding to an immunorecessive self-Ag epitope would be affected by tolerance in much the same manner as TCDS responding to immunodominant epitopes. Such mechanisms could include anergy and/or deletion. Our data demonstrate that TCR-V T cells acquire effector function in response to the endogenous Tag. It remains possible that prolonged exposure to endogenous Tag renders TCR-V cells destined for future deletion because these cells are not retained at detectable levels unless the mice are immunized. Similar observations have been made with TCR-transgenic T cells responding to immunodominant tumor epitopes upon transfer into tumor-bearing mice (34, 47, 57). Our findings here using TCR-transgenic T cells imply that higher avidity endogenous epitope V specific TCDS that survive negative selection in SV11 mice might additionally be subject to tolerance upon recognition of and activation by persistent Tag in the peripheral tissues, thereby contributing to the low levels of functional epitope V-specific T cells available for recruitment in tumor-bearing mice (17). Whether the endogenous epitope V-specific T cells isolated from SV11 mice have avidities similar to the TCR-V T cells has not been determined. If so, their lasting presence might require continued export of new cells from the thymus (58).

We found that immunization of SV11 mice extends the expansion phase of TCR-V cells, but does not increase the magnitude to that observed in B6 mice. There are several possible explanations for the lower total accumulation of TCR-V cells in SV11 mice vs B6 mice following immunization. First, our kinetic analysis revealed that adoptively transferred cells can be triggered by epitope V derived from endogenous Tag 24–48 h before the effects of immunization are realized. A similar lag in response to immunization has been observed in other systems (59–61) and may correlate with the time required for APCs to localize to secondary lymphoid organs (62). Thus, TCDS that have encountered endogenous Tag may initially be refractory to rapid reactivation by exogenous Tag delivered on the day of adoptive transfer due to the induction of activation-induced inhibitory pathways (63). Alternatively, there could be fewer TCDS available for exposure to Tag derived from immunization if the cells have migrated to a more distant tissue. In fact, we found that TCR-V T cells accumulate rapidly in the CLN following transfer into SV11 mice, distant from the immunization site in the peritoneal cavity. Another possibility is that some of the transferred TCR-V T cells are irreversibly inactivated by recognition of the endogenous Tag, due to the immediate and persistent expression of Tag. Immunization of SV11 mice provides an additional source of Ag outside of the CLN that might convey a qualitatively different signal from that delivered by Ag draining from the tumor site. It has been postulated that the nature of the initial encounter with APC can program the activation, expansion, function, and ultimate survival of naive TCDS (64, 65). Potentially, an APC that displays endogenously derived Tag-V originating from the brain might deliver a tolerogenic signal to the naive TCR-V cell it engages. Meanwhile, an APC that cross-presents exogenously derived Tag-V might confer a contrasting survival signal. Whether direct interaction with B6/V-only Tag cells may rescue TCR-V cells that have been partially activated by endogenous Tag remains unknown.

Our data suggest that the migration of TCR-V cells to the brains of SV11 and B6 mice is not initially Ag dependent following primary immunization. Rather, the initial migration into the brain reflects the presence of activated cells in the periphery. A similar observation was made previously for TCDS responding to the dominant Tag epitope IV (31). Entry into the CNS has been reported to require the expression of activation molecules such as CD43 (66) in addition to the up-regulation of specific homing receptors (67, 68). Extravasation is subsequently dependent upon interaction of these receptors with cognate-binding partners on the brain vasculature, including ICAM, VCAM, PECAM, and P-selectin. Preliminary data has shown that a

<table>
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<th>Treatment</th>
<th>Median survival (days)</th>
<th>P value</th>
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<tbody>
<tr>
<td>no treatment</td>
<td>102</td>
<td>-</td>
</tr>
<tr>
<td>TCR-V only</td>
<td>101</td>
<td>0.3486</td>
</tr>
<tr>
<td>TCR-V + prime (Tag-V only)</td>
<td>104</td>
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<tr>
<td>primer/boost only (Tag-V only)</td>
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</tr>
</tbody>
</table>

FIGURE 7. Prime and boost promotes increased survival of tumor-bearing SV11 mice. The lifespans of tumor-bearing 85-day-old SV11 mice treated with the indicated therapeutic treatments were compared. Immunizations (prime and boost) consisted of B6/Tag-V only cells or cells expressing a variant Tag in which epitopes I, III, IV and V were inactivated (Tag epitope-null). ***, Significantly different from group receiving no treatment by log-rank test.
subset of TCR-V cells recovered from the spleens of immunized SV11 and B6 mice express CD38, the lymphocyte receptor for the endothelial ligand PECAM (data not shown). Furthermore, a homogeneous population of brain-isolated TCR-V cells had up-regulated this homing molecule (data not shown).

In this study, the booster immunization drove a dramatic accumulation of TCR-V cells in the brains of SV11 but not B6 mice. A possible explanation for this finding is that the booster immunization leads to an initial expansion of Ag experienced TCR-V cells in the peripheral lymphoid organs, as evidenced by a dramatic increase in the frequency of TCR-V T cells in the spleens of B6 mice. In SV11 mice, these cells might rapidly migrate to and accumulate in the large choroid plexus tumors. In B6 mice, these cells likely migrate through the brain and back into the circulation in the absence of specific Ag. Whether the large accumulation of TCR-V T cells in the brains of SV11 mice that received a booster immunization is due to the influx of cells from the periphery or represents local expansion within the tumor remains to be determined.

The effect of the booster immunization in SV11 mice was quite dramatic compared with the effect of the primary immunization de- spite the presence of a large number of precursor cells within the lymphoid organs at the time of initial immunization. Our data indicate that the majority of transferred TCR-V cells are rapidly triggered following exposure to the endogenous Tag in SV11 mice and most likely remain in an activated state 24–48 h after transfer. Thus, they might be unable to efficiently respond to the B6/V-only Tag immunization administered on the same day. However, following contraction at day 5, they are better able to respond to the B6/V-only Tag booster administered on day 7. Suboptimal activation of T<sub>CD8</sub> has been shown to occur in vivo, leading to a T cell population that, while Ag experienced and capable of IFN-γ production, remains undivided (69). However, this incompletely differentiated cell population responded to secondary challenge. Therefore, TCR-V cells that receive a suboptimal signal after stimulation by the endogenous Tag might become fully activated and migrate to the tumor upon delivery of the day 7 booster immunization.

Despite the vigorous enhancement of epitope V-specific T<sub>CD8</sub> at the tumor site and significant increase in survival, all mice eventually succumbed to tumor burden. Preliminary evidence indicates that the frequency of tumor resident TCR-V cells declines significantly in primed and boosted SV11 mice by the time of death (data not shown). Thus, it is possible that TCR-V T cells are eventually subject to the same tolerogenic mechanisms as those observed in immunodominant epitope-specific T cell models. A similar transient effect was observed in SV11 mice following the activation of adoptively transferred normal B6 spleen cells against the immunodominant epitope Tag IV by specific immunization (26). Common obstacles to T<sub>CD8</sub>-mediated tumor immunotherapy include alterations at the tumor site itself, including 1) the presence of T regulatory cells, 2) tumor cell down-regulation of MHC class I expression, 3) the emergence of tumor cell escape variants that cease to express the epitopes recognized by their cognate tumor-reactive T cells, and 4) production of T cell suppressive cytokines by the tumor (40, 70). Expression of the anti-inflammatory cytokine TGF-β has been reported in SV11 mice with progressing tumors (71). Therefore, it is possible that eventually TCR-V cells are rendered nonfunctional by the tumor microenvironment. Thus, it will be important to address the state of the tumor microenvironment to investigate whether similar mechanisms of immune evasion occur in response to epitope V-specific T cells.

Despite decades of intense research, cancer remains an enigmatic challenge for the immune system. One of the greatest obstacles in patients with tumors that express self-Ag epitopes is the paucity of T<sub>CD8</sub> that harbor potent tumor reactivity. We have attempted to closely mirror the human cancer scenario using the SV11 mouse model, and report a quantitative and qualitative study monitoring the fate of T<sub>CD8</sub> specific for an immunorecessive tumor epitope in the context of a spontaneously arising tumor. Our findings that TCR-V cells recognize endogenous Tag and respond to immunization in a manner that leads to tumor infiltration and prolonged the survival of SV11 mice sets a promising backdrop for continued investigations that target T cells specific for similar subdominant or immunorecessive epitopes. The exploration of strategies that lead to sustained T<sub>CD8</sub> responses in this and similar models will provide important preclinical clues toward developing immunotherapeutic approaches that succeed in the treatment of cancer.

**Acknowledgments**

We thank Dr. Pavel Otahal for assistance with confocal imaging and Jeremy Haley and Melanie Eppler for excellent technical support. We also thank Dr. Satvir Tevethia for critical reading of the manuscript and Nate Schaffer from the Flow Cytometric Core Facility of the M. S. Hershey Medical Center for assistance with flow cytometry.

**Disclosures**

The authors have no financial conflict of interest.

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