Early Immunization Induces Persistent Tumor-Infiltrating CD8$^+$ T Cells against an Immunodominant Epitope and Promotes Lifelong Control of Pancreatic Tumor Progression in SV40 Tumor Antigen Transgenic Mice[1]

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The ability to recruit the host's CD8$^+$ T lymphocytes (T_{CD8}) against cancer is often limited by the development of peripheral tolerance toward the dominant tumor-associated Ags. Because multiple epitopes derived from a given tumor Ag (T Ag) can be targeted by T_{CD8}, vaccine approaches should be directed toward those T_{CD8} that are more likely to survive under conditions of persistent Ag expression. In this study, we investigated the effect of peripheral tolerance on the endogenous T_{CD8} response toward two epitopes, designated epitopes I and IV, from the SV40 large T Ag. Using rat insulin promoter (RIP) 1-Tag4 transgenic mice that express T Ag from the RIP and develop pancreatic insulinomas, we demonstrate that epitope IV- but not epitope I-specific T_{CD8} are maintained long term in tumor-bearing RIP1-Tag4 mice. Even large numbers of TCR-transgenic T cells specific for epitope I were rapidly eliminated from RIP1-Tag4 mice after adoptive transfer and recognition of the endogenous T Ag. Importantly, immunization of RIP1-Tag4 mice at 5 wk of age against epitope IV resulted in complete protection from tumor progression over a 2-year period despite continued expression of T Ag in the pancreas. This extensive control of tumor progression was associated with the persistence of functional epitope IV-specific T_{CD8} within the pancreas for the lifetime of the mice without the development of diabetes. This study indicates that an equilibrium is reached in which immune surveillance for spontaneous cancer can be achieved for the lifespan of the host while maintaining normal organ function. ***The Journal of Immunology,*** **2006, 177: 3089–3099.**

A variety of experimental models have been used to examine the role of CD8$^+$ T lymphocytes (T_{CD8})[5] in the immunotherapy of tumors. These models include transplantable tumors as well as spontaneously arising tumors that develop in transgenic mice expressing cell- or virus-derived oncogenes. Such studies have revealed a multitude of factors that may inhibit an effective T_{CD8}-mediated response against the tumor (reviewed in Ref. 1), including but not limited to the deletion of tumor-specific T_{CD8} by central tolerance (2–5) or their inactivation by peripheral tolerance (6–8). Mechanisms that allow the tumor to resist even an active T_{CD8} response also contribute to failed immunotherapy of cancer, such as the inability of tumor-specific T_{CD8} to access the tumor (9). Some experimental models have used surrogate tumor Ags (T Ags) expressed in transplantable and transgene-induced tumors to monitor the role of endogenous T_{CD8} in the control of tumor growth (5, 10–12) or, alternatively, have assessed the response of T_{CD8} derived from TCR transgenic mice toward transplantable or oncogene-induced spontaneous tumors (13–17). Few studies, however, have assessed the role of the endogenous natural T_{CD8} for the control of spontaneous tumors expressing a known tumor Ag (18–22).

T_{CD8} recognize peptides bound to MHC class I molecules on the tumor cell surface. These peptides are derived from the processing of intracellular proteins, with each MHC class I allele binding different combinations of the available peptides for presentation at the cell surface (23). Thus, a wide variety of peptide/MHC complexes is presented at the tumor cell surface, and the tumor-specific T_{CD8} response can be targeted against multiple epitopes simultaneously, although with varying efficiencies (24). This phenomenon, known as immunodominance, is controlled by multiple factors affecting Ag processing and presentation as well as the T cell repertoire of the host (25). Because multiple tumor epitopes could potentially be targeted, assessing the nature of the immune response toward epitopes that lead to the most effective control of tumor progression in experimental models will provide clues toward better approaches to the immunotherapy of cancer.

Our previous studies using two different transgenic mouse lines expressing SV40 large tumor Ag (T Ag) indicate that immunodominance among T Ag epitopes contributes toward the efficiency of T_{CD8}-mediated control of progressively growing tumors

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[5] Abbreviations used in this paper: T_{CD8}, CD8$^+$ T lymphocyte; B6, C57BL/6; Db, H-2D^b; Db/I, Db epitope I; Flu, influenza virus; gB, glycoprotein B; HA, hemagglutinin; Kb, H-2K^b; Kb/IV, Kb epitope IV; LCMV, lymphocytic choriomeningitis virus; NP, nucleoprotein; RIP, rat insulin promoter; T Ag, tumor Ag.

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due to differential inactivation of T Ag-specific T_{CD8} (3, 6). SV40 T Ag encodes four H-2^b-restricted epitopes (26). Epitopes I (residues 206–215), II/III (residues 223–231), and V (residues 489–497) are H-$2D^b$ (Db)-restricted, whereas epitope IV (residues 404–411) is H-$2K^b$ (Kb)-restricted. These four T Ag-derived, T_{CD8}-recognized epitopes fall on a hierarchical scale, with epitope IV being the most immunodominant followed by epitopes I and II/III (27, 28). Epitope V is immunorecessive and not immunogenic unless the three dominant epitopes are inactivated in T Ag (29, 30). The ability of T_{CD8}-specific for T Ag to control tumor progression was established by showing that the transfer of T_{CD8} specific for the immunodominant T Ag epitopes into SV11 T Ag transgenic mice induced regression of advanced stage spontaneous tumors (31).

To better understand the role of the endogenous T_{CD8} repertoire in the control of spontaneous tumor progression, we took advantage of the well-characterized tumor model of rat insulin promoter (RIP) 1-Tag4 transgenic mice on the C57BL/6 (B6) background (32). In RIP1-Tag4 mice, T Ag is expressed as a transgene from the rat insulin II promoter in pancreatic β cells, which leads to highly predictable formation of β cell tumors. The transformation of β cells by T Ag is a gradual process, initially inducing development of islet hyperplasia by three months of age and followed by the appearance of macroscopic insulinomas between 5 and 6 mo of age (18, 33). The average life span of RIP1-Tag4 mice is ~200 days, with 100% of mice developing tumors. Transformed β cells secrete high amounts of insulin leading to the development of hypoglycemic shock and death of tumor-bearing mice. Previously, Knowles and colleagues (18) demonstrated that immunization of RIP1-Tag4 mice with SV40 before or during T Ag-induced tumorigenesis resulted in the development of T_{CD8} capable of lysing syngeneic T Ag-transformed cells in vitro. However, only immunization before the expression of T Ag in the β cells (at 3 wk of age) led to long-term protection against tumor development. Whether the composition of T Ag-specific T_{CD8} response was altered by expression of the endogenous T Ag was not addressed. Although this previous study demonstrated that the presence of T Ag-specific T_{CD8} is associated with long-term control of tumor progression in RIP1-Tag4 mice, it remained to be determined whether peripheral tolerance limits T_{CD8} responding to individual T Ag epitopes over the course of tumor progression and whether long-term protection from tumor progression is associated with a particular T Ag epitope.

Our results show that activation of endogenous T_{CD8} specific for the dominant SV40 T Ag epitope IV before T Ag expression in the pancreatic β cells leads to lifelong surveillance against T Ag-induced spontaneous tumors. Importantly, functional epitope IV-specific but not epitope I-specific T_{CD8} persist in the host without inducing tissue destruction or diabetes in RIP1-Tag4 mice.

Materials and Methods

Mice

B6 (H-2^b) mice were purchased from The Jackson Laboratory, and B6.SJL mice (CD45.1^+) were procured from Taconic Farms. Line RIP1-Tag4 mice are on the B6 background and express the SV40 early region as a transgene under control of the rat insulin promoter and were described previously (32). TCR-I mice express epitope I-specific TCRα and TCRβ-chains as a transgene (15) and are available from The Jackson Laboratory as line B6.Cg-Tg(Tcra TcrB)416Tev/J. Approximately 95% of T_{CD8} express the TCR transgenes. All mice were maintained at the animal facility of the Milton S. Hershey Medical Center (Hershey, PA). Animal studies were performed in accordance with guidelines established by the Pennsylvania State University College of Medicine Institutional Animal Care and Use Committee (Hershey, PA) under an approved protocol.

Reagents and immunizations

The B6/WT-19 cell line was derived previously by transformation of B6 mouse embryo fibroblasts with wild-type SV40 strain VA45-54 (34). B6/15Bb cells express a T Ag variant in which epitopes I, II/III, and V have been inactivated by site-directed mutagenesis but which encodes wild-type epitope IV (27). Cell lines were maintained in DMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin, 2 μM L-glutamine, 10 mM HEPES, 0.075% (w/v) $NaHCO_3$, and 5–10% FBS. Peptides were synthesized at the Macromolecular Core Facility of the Milton S. Hershey Medical Center by Fmoc chemistry using an automated peptide synthesizer (9050 MilliGen PepSynthesizer; Millipore). Peptides used in these experiments correspond to the SV40 T Ag epitope I (SAIN NYAQKL; 206–215), epitope IV containing a Leu replacement of Cys at residue 411 (VVYDFLKL; 404–411), and an epitope from HSV glycoprotein B (gB) (SSIEFARL; 498–505). Immunization of mice with T Ag-expressing cell lines was conducted by i.p. injection of 3–5 $\times$ 10^7 cells in 0.5 ml of HBSS at the indicated ages.

Flow cytometric analysis and in vitro stimulation of bulk CTL cultures

Ex vivo staining of T_{CD8} with MHC tetramers and primary conjugated Abs was performed on single-cell suspensions prepared from spleens as described previously (27). For dissociation of tumors and pancreas, tissue was minced with a razor blade and incubated in HBSS containing 1% FBS, 1 mg/ml collagenase D (Roche), and 50 U/ml DNase I (Roche) for 1 h at 37°C. Any remaining clumps were dissociated by gentle pipetting, and cells were washed before tetramer staining. In some cases, spleen cells were cultured in vitro for 6 days in the presence of γ-irradiated B6/WT-19 stimulator cells as described previously (3). After staining, cells were fixed with 2% paraformaldehyde in PBS and analyzed using a FACScan or FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (Tree Star). Production and characterization of the Db/T Ag epitope I (Db/I), Kb/T Ag epitope IV (Kb/IV), Db/influenza virus (Flu) nucleoprotein (NP) epitope 366–374 (Db/Flu), and Kb/HSV gB epitope 498–505 (Kb/gB) tetramers were described previously (27). The following mAbs were purchased from BD Pharmingen: PE- and CyChrome-labeled rat anti-CD8α (clone 53-6-7), FITC-labeled rat anti-mouse IFN-γ (clone XMG1.2), and PE-labeled anti-mouse CD45.1 (clone A20). The percentage of $CD8^+$ cells that stained positive for the T Ag-specific tetramer was determined by subtracting the percentage of cells that stained positive with the control Db/Flu or Kb/gB tetramers within the same population.

Adoptive transfer and proliferation assay

Adoptive transfers were performed using sex-matched, RBC-depleted lymphocytes derived from spleens and lymph nodes of TCR-I transgenic mice. Recipient mice received i.v. injections of lymphocytes derived from spleen and lymph nodes of TCR-I mice that contained 1–2 $\times$ 10^6 clonotypic $CD8^+$ T cells as determined by prior staining with MHC tetramers. Mice were immunized the following day. For proliferation assays, TCR-I-derived lymphocytes were labeled with 5 μM CFSE (Invitrogen Life Sciences) for 10 min at 37° C in PBS and 0.1% BSA followed by three washes before i.v. injection into recipient mice. Proliferating cells were detected after three days by measuring the mean fluorescent intensity of CFSE staining retained in $CD8^+$ Db/I tetramer-positive cells recovered from the spleens of recipient mice.

Intracellular cytokine assays

For staining of intracellular IFN-γ, RBC-depleted lymphocyte suspensions were prepared as described above and incubated at 5 $\times$ 10^6/ml with the indicated synthetic peptides (1 μM) representing T Ag or control epitopes and 1 μg/ml brefeldin A in complete RPMI 1640 containing 10% FBS for 4 h at 37°C in 5% CO_2. The Cytofix/Cytoperm kit (BD Pharmingen) was used to stain for IFN-γ production according to the manufacturer's instructions and as described previously (27). For peptide titration assays, splenocytes from B6/WT-19-immunized mice were restimulated in vitro with γ-irradiated B6/WT-19 cells for 5 days at 37° C with 5% CO_2. Responder cells were plated at 5 $\times$ 10^5 cells/well in the presence of titrated amounts of the epitope IV or control K^b-binding peptide 498–505 from HSV gB (35) and 1 μg/ml brefeldin A. After a 6-h incubation at 37°C with 5% CO_2, cultures were stained as described above.

In vivo cytotoxicity assay

Splenocytes from B6.SJL mice (CD45.1^+) were incubated in the presence of the indicated peptides (1 μM) in RPMI 1640 with10% FBS at 37°C for 90 min and washed three times to remove excess peptide. Peptide-coated

targets were then labeled with varying concentrations (5 μM CFSE^{high}, 0.5 μM CFSE^{medium}, and 0.025 μM CFSE^{low}) of CFSE (Invitrogen Life Sciences) for 10 min at 37°C in PBS and 0.1% BSA, washed 2 times, and mixed together at 1:1:1 ratio, and then 6×10^6 cells were injected i.v. into the tail vein of sex-matched mice in 0.2 ml HBSS. The elimination of CFSE-labeled targets was assessed after 16 h by analyzing spleen cells for the presence of CD45.1$^+$CFSE$^+$ cells. The following formula was used: percentage of specific killing = [1 − (ratio of control mice/ratio of immunized mice) × 100], where ratio equals the percentage of CFSE^{low}/CFSE$^{high\ or\ medium}$. CFSE^{low} targets were pulsed with control peptide gB and used as an internal control for nonspecific lysis.

Tumor histology, immunohistochemistry, and measurement of islet size

For T Ag immunohistochemistry, pancreata were fixed with 10% buffered formalin before being embedded in paraffin blocks. Eight-micrometer-thick sections were cut on a microtome and collected onto positively charged slides. Sections were deparaffinized in xylene and rehydrated in ethanol. After two washes in PBS, slides were treated with Ag-unmasking solution (Vector Laboratories) for 30 min in a steamer. Cooled slides were washed in PBS, incubated with 10% normal goat serum in PBS containing 0.1% Tween 20 for 30 min, and washed and incubated with primary anti-T Ag Ab for 1 h at room temperature. Primary Ab consisted of an equal mixture of mAb Pab419 (36) and Pab901 (37), culture supernatants that bind to epitopes in the amino-terminal and carboxyl-terminal portions of SV40 T Ag, respectively. Sections were then incubated for 1 h with biotinylated anti-mouse Ab and visualized with peroxidase/diaminobenzidine detection (ChemMate detection kit; Ventana) for 2–10 min, as needed. Parallel sections were stained with H&E. Sections were examined using a Nikon Microphot-FXA microscope, and representative images were captured using a Sony DKC-ST5 digital camera. To measure the size of individual islets, H&E-stained pancreatic sections were examined under the microscope using a micrometer. The radius of all islets per section was measured, and their individual sizes were plotted. Ten sections were examined for each mouse.

Life span analysis and measurement of blood glucose levels

Mice in life span analyses were monitored for symptoms of hypoglycemia, which are indicative of advanced stage tumors, and sacrificed when blood glucose levels dropped below 50 mg/dl. Blood glucose levels were measured using an Elite glucometer (Bayer). Kaplan-Meier survival plots were constructed using Prism software (GraphPad).

Results

RIP1-Tag4 mice with progressively growing tumors remain responsive to T Ag epitope IV but not to T Ag epitope I

To determine whether T_{CD8} specific for the immunodominant T Ag epitopes remain sensitive to immunization in aged RIP1-Tag4 mice, groups of three RIP1-Tag4 mice aged 35, 90, and 180 days as well as 90-day-old B6 mice were immunized i.p. with T Ag-transformed B6/WT-19 cells that express wild-type T Ag. Nine days later, splenic T_{CD8} lymphocytes were stained with anti-CD8 Ab and MHC class I tetramers capable of detecting T cells specific for epitope I (Db/I) or epitope IV (Kb/IV). A tetramer composed of the influenza NP epitope $NP_{366-374}$ (Db/Flu) was used as a control tetramer. The data in Fig. 1*A* show that epitope IV-specific T cells expanded in 35-day-old RIP1-Tag4 mice to levels similar to those for the control B6 mice. The number of epitope IV-specific T_{CD8} recruited by immunization gradually declined in older RIP1-Tag4 mice but remained significant even at 180 days of age. In contrast, the T_{CD8} response against epitope I already was decreased in 35-day old RIP1-Tag4 mice compared with B6 mice and was undetectable by tetramer staining in 180-day-old RIP1-Tag4 mice.

We determined whether T Ag-specific T_{CD8} recruited by immunization with B6/WT-19 cells localized to the tumors in older RIP1-Tag4 mice. Groups of 180-day-old RIP1-Tag4 mice were immunized with B6/WT-19 cells. Nine days later, tumors were enzymatically digested and epitope-specific T_{CD8} were directly enumerated by staining with MHC tetramers. The data in Fig. 1*B* show that epitope IV-specific T_{CD8} (29% of total T_{CD8}) infiltrated the pancreatic tumors. In parallel experiments we found that approximately half the number of Kb/IV tetramer-positive T_{CD8} detected in the tumor at this time point were capable of producing IFN-γ in response to short-term incubation with epitope IV peptide as determined by intracellular cytokine staining (data not shown). Staining of tumor-derived T_{CD8} with the Db/I tetramer did not show positive cells above the background staining obtained with control Db/Flu tetramer. These results show that a proportion of epitope IV-specific but not epitope I-specific T_{CD8} survive peripheral tolerance in tumor-bearing RIP1-Tag4 mice and remain responsive to immunization, resulting in the infiltration of pancreatic tumors.

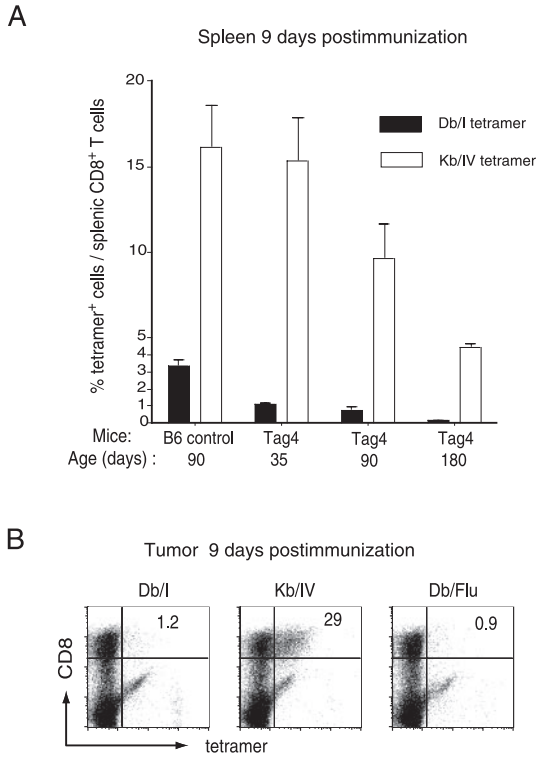

FIGURE 1. RIP1-Tag4 mice develop tolerance to T Ag epitope I but not epitope IV. *A*, Groups of three RIP1-Tag4 mice aged 35, 90, and 180 days and control 90-day-old B6 mice were immunized i.p. with wild-type T Ag-transformed B6/WT-19 cells. Nine days later the frequency of epitope I- and epitope IV-specific T_{CD8} was determined by tetramer staining of spleen cells. The values obtained with a control Db/Flu tetramer specific for influenza $NP_{366-374}$ were subtracted to obtain the percentage of epitope-specific T cells per total T_{CD8}. *B*, Epitope IV-specific but not epitope I-specific T_{CD8} are detected in pancreatic tumors following immunization. RIP1-Tag4 mice aged 180 days were immunized i.p. with WT-19 cells, and 9 days later the frequency of epitope I- and epitope IV-specific T_{CD8} was determined by tetramer staining of the resulting cell suspension prepared from pancreatic tumors using enzymatic digestion. Control Db/Flu tetramer was used to determine the specificity of staining. Values in the *upper right quadrant* indicate the percentage of total T_{CD8} that are tetramer positive. Similar results were obtained in three independent experiments with 2–3 mice per group.

Epitope I-specific TCR transgenic T cells are rapidly tolerized in RIP1-Tag4 mice

Following T Ag immunization of 5-wk-old naive RIP1-Tag4 mice, the response to epitope IV is ~15-fold greater than that directed toward epitope I (Fig. 1). Whether this difference can be traced to fewer precursors in the naive T cell repertoire targeting epitope I

vs epitope IV is unknown. If such a discrepancy exists, however, the time required to induce tolerance among epitope I-specific T_{CD8} vs epitope IV-specific T_{CD8} may be significantly shorter. To determine whether an increased frequency of epitope I-specific T_{CD8} would lead to a more enduring response in RIP1-Tag4 mice, we used adoptive transfer of naive T_{CD8} from epitope I-specific TCR transgenic mice in which >95% of the T_{CD8} are specific for epitope I (15). In a previous study, TCR-I transgenic T cells were shown to be triggered by the endogenous T Ag after transfer into mice expressing T Ag as a transgene from the α-amylase promoter (15). To monitor the consequences of TCR-I T cell transfer into RIP1-Tag4 mice, CFSE-labeled TCR-I-derived lymphocytes were transferred into RIP1-Tag4 mice at various ages representing the various stages of tumor progression. The ability of the transferred T_{CD8} to proliferate in response to the endogenous T Ag was monitored after 3 days. The frequency of TCR-I cells detected in the spleens of RIP1-Tag4 mice increased slightly over that detected in B6 mice, with the highest number of cells detected in 6-mo-old RIP1-Tag4 mice (Fig. 2*A*, *upper panels*). This increase corresponded with the presence of proliferating cells in the RIP1-Tag4 mice (Fig. 2*A*, *bottom panels*). Although TCR-I T cells initially proliferated in RIP1-Tag4 mice, these cells were rapidly eliminated by day 10 as evidenced by only minimal detection in the spleen (Fig. 2*B*) and lack of detection in the pancreas (data not

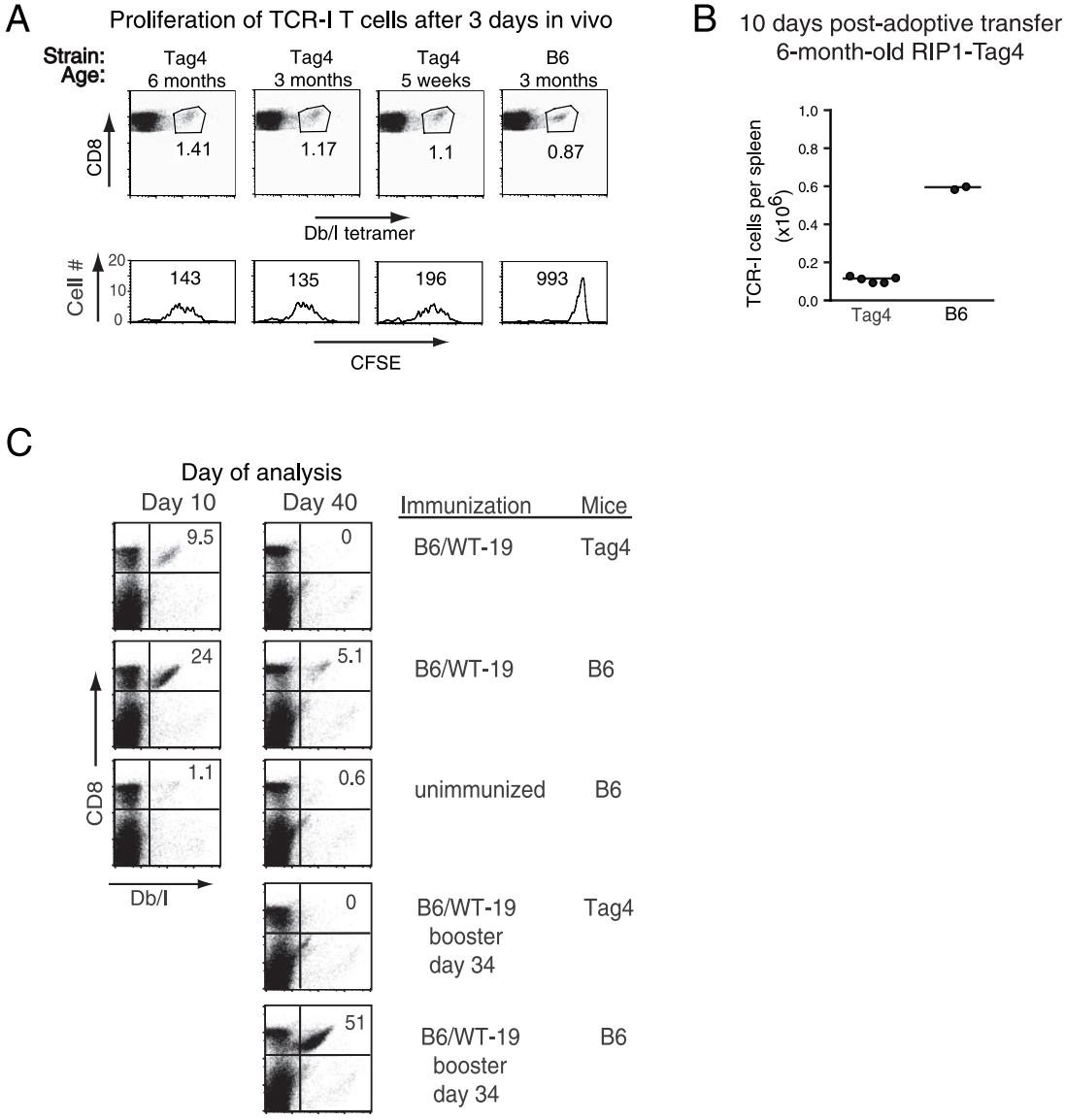

FIGURE 2. Epitope I-specific T_{CD8} are rapidly deleted from RIP1-Tag4 mice. *A*, RIP1-Tag4 (Tag4) and B6 mice were injected i.v. with 1×10^6 CFSE-labeled clonotypic TCR-I T cells. After 3 days, lymphocytes were recovered from spleens of recipient mice and stained with anti-CD8 and Db/I tetramer. The frequency of $CD8^+$ cells specific for epitope I is indicated in the dot plots and have been corrected by subtraction of the frequency of cells that stained positively with a control tetramer. The histograms show the mean fluorescence intensity of CFSE detected in $CD8^+$ Db/I tetramer-positive cells. Results for individual mice are shown, and similar results were obtained in two mice for each group. *B*, Twenty million clonotype-specific TCR-I T cells were injected i.v. into 6-mo-old RIP1-Tag4 or control B6 mice. After 10 days, spleen cells were analyzed for the presence of $CD8^+$ Db/I tetramer-positive cells. The total number of epitope I-specific T_{CD8} per individual spleen is indicated, with the horizontal bars representing the mean. *C*, Groups of 3-mo-old RIP1-Tag4 and B6 mice received 1×10^6 clonotypic TCR-I cells i.v. followed by no immunization or immunization the next day with 3×10^7 B6/WT-19 cells. Splenocytes were harvested from recipient mice at 10 or 40 days after adoptive transfer and stained for the presence of $CD8^+$ Db/I tetramer-positive cells. Some mice received a booster immunization with B6/WT-19 cells on day 34 after adoptive transfer. The values represent the percentage of $CD8^+$ cells specific for epitope I and have been corrected by subtraction of the percentage of cells that stained positively with a control tetramer. The plots represent data collected from individual mice with two mice per group showing similar results.

shown) of 6-mo-old mice. Similar results were obtained when TCR-I cells were transferred into younger mice (data not shown). These results indicate that epitope I-specific TCR transgenic T_{CD8} are rapidly activated and eliminated from RIP1-Tag4 hosts following recognition of the endogenous T Ag, consistent with loss of the endogenous population of epitope I-specific T_{CD8}.

We next determined whether immunization could delay the loss of TCR-I T cells from RIP1-Tag4 hosts. At 3 mo of age, epitope I-specific T_{CD8} represent ~1% of splenic T_{CD8} following immunization of RIP1-Tag4 mice (Fig. 1*A*). This level was increased to 9.5% of T_{CD8} following adoptive transfer with TCR-I cells and subsequent immunization (Fig. 2*C*), a level comparable to that of the epitope IV-specific T_{CD8} that accumulate from the endogenous T cell repertoire (Fig. 1*A*; 3 mo). We noted that 2.5-fold more epitope I-specific T_{CD8} accumulated in the spleens of B6 mice compared with RIP1-Tag4 mice following adoptive transfer of TCR-I cells and immunization (Fig. 2*C*). By 40 days postimmunization, epitope I-specific T_{CD8} had decreased below the limit of detection by tetramer analysis in RIP1-Tag4 mice but represented 5% of $CD8^+$ cells in B6 mice, a 5-fold decrease from day 10. To determine whether a residual population of TCR-I T cells was present in RIP1-Tag4 mice at this later time point, some mice were given a booster immunization with B6/WT-19 cells at day 34, 6 days before analysis. The results demonstrate that epitope I-specific T_{CD8} remained undetectable in RIP1-Tag4 mice despite extensive expansion in B6 mice. Thus, immunization successfully induces the expansion of TCR-I T cells in RIP1-Tag4 mice leading to initial accumulation at levels similar to that obtained with the endogenous epitope IV-specific T_{CD8}, but these T_{CD8} fail to persist long term despite this increased frequency.

Early immunization of RIP1-Tag4 mice against epitope IV prevents the development of tumors without inducing diabetes

Because we observed that T_{CD8} specific for epitope IV but not epitope I could be triggered even in tumor-bearing RIP1-Tag4 mice (Fig. 1*A*), we determined whether specific immunization against the immunodominant epitope IV alone could mediate control of tumor progression in RIP1-Tag4 mice. A group of 35-day-old RIP1-Tag4 mice were immunized i.p. with B6/15Bb cells expressing a T Ag variant (epitope IV-only) containing epitope IV but lacking the other H-2^b-restricted epitopes I, II/III, and V (27). Immunization with B6/15Bb cells results in a quantitatively similar T_{CD8} response to epitope IV as observed following immunization with B6/WT-19 cells expressing the wild-type T Ag (data not shown). Another group of RIP1-Tag4 mice was immunized at 180 days of age and survival was monitored. The data in Fig. 3*A* demonstrate that early immunization at 35 days of age with epitope IV-only T Ag-expressing cells prevented the progression of tumors, because all mice in the group ($n = 8$) lived for two years, at which time these mice were euthanized for further analysis (see below). In contrast, immunization of RIP1-Tag4 mice with epitope IV-only T Ag-expressing cells at 180 days of age was not effective at controlling tumor progression ($n = 8$, median 204 days), and their life span was similar to untreated RIP1-Tag4 mice ($n = 10$, median 194 days). Thus, immunization of RIP1-Tag4 mice at 35 days of age against epitope IV results in a normal disease-free life span, whereas immunization of tumor-bearing mice had a minimal effect on survival.

To monitor the effect of immunization on tumor growth, we measured the size of individual islets at various ages in RIP1-Tag4 mice that remained unimmunized (Fig. 3*C*) or were immunized with IV-only T Ag-expressing cells at 35 days of age (Fig. 3*D*). Increased islet size in unimmunized RIP1-Tag4 mice can be detected as early as 84 days of age, which is consistent with the reported development of initial islet hyperplasia (38). Islet size further increased by 175 days of age, corresponding with the appearance of macroscopic tumors. Histological analysis on days 180 and 720 of pancreata from immunized RIP1-Tag4 mice (Fig. 3*D*) revealed only modest hyperplasia with islet sizes similar to that found in normal B6 mice, although small tumors could be detected in some mice (1–1.5-mm in diameter). Thus, the protective effect of early immunization against epitope IV correlates with inhibition of tumor development in RIP1-Tag4 mice.

Immunization against the T Ag might be expected to induce an autoimmune phenotype and subsequent diabetes if β cells are specifically destroyed by responding immune cells as has been observed in similar transgenic models (39, 40). However, we found that mice immunized with B6/15Bb cells at 5 wk of age that survived long term maintained normal levels of blood glucose (Fig. 3*B*), indicative of continued β cell function. In contrast, naive RIP1-Tag4 mice became hypoglycemic by 27 wk of age, consistent with the progression of β cell tumors producing high levels of

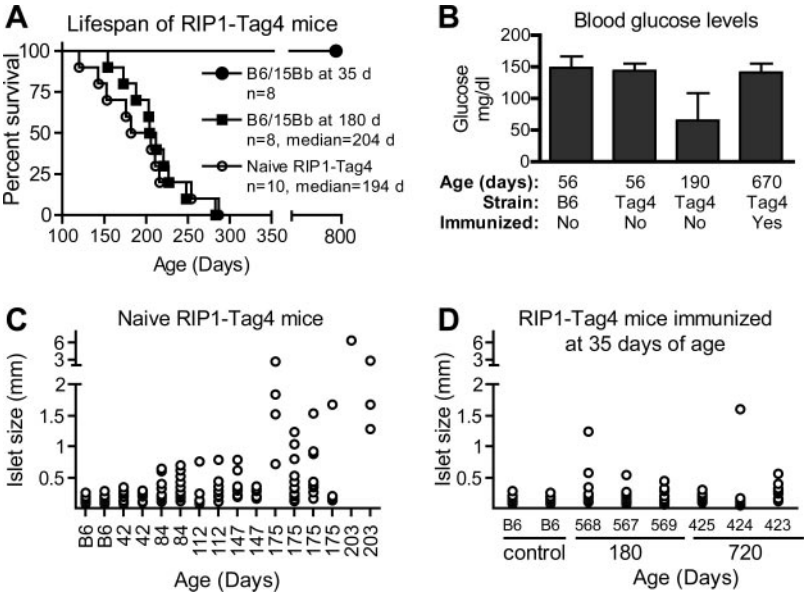

FIGURE 3. Immunization against epitope IV induces lifelong protection from tumor progression in RIP1-Tag4 mice. *A*, Groups of RIP1-Tag4 mice were immunized with T Ag-transformed B6/15Bb cells expressing a T Ag variant containing only epitope IV but not epitopes I, II/III, and V at 35 (median survival >2 years) or 150 days of age (median survival, 204 days). A third group remained unimmunized (median survival, 194 days). *B*, Blood glucose levels were measured at the indicated times in naive mice or in RIP1-Tag4 mice that had been immunized with B6/15Bb cells at 5 wk of age. Values represent the mean glucose levels ± SE for 6–8 mice. *C* and *D*, The pancreatic islet size was measured using H&E-stained pancreatic sections prepared from untreated RIP1-Tag4 mice (*C*) or RIP1-Tag4 mice immunized at 35 days with B6/15Bb cells (*D*). Data in each column show the size of individual islets in each mouse, represented by the open circles. Islet sizes from untreated B6 mice are shown for comparison.

insulin (32). Thus, immunization against T Ag epitope IV results in continuous and long-term protection from tumor progression without disrupting pancreatic function.

Immunized RIP1-Tag4 mice survive long term despite continued expression of T Ag

Long-term survival of RIP1-Tag4 mice immunized with T Ag-transformed cells at 35 days of age could be explained by the loss of β cells expressing the T Ag transgene. To assess this possibility, we determined whether long-term surviving RIP1-Tag4 mice that were immunized at 35 days of age maintain T Ag expression in the pancreatic islets as detected by immunohistochemistry (Fig. 4). Islet cells from unimmunized B6 mice lacked T Ag expression (Fig. 4*C*), whereas the majority of cells in the islets of 6-mo-old naive RIP1-Tag4 mice were positive for T Ag expression (Fig. 4*D*). We then analyzed pancreatic tissue obtained from long-term surviving RIP1-Tag4 mice that were immunized at 35 days of age. The histological analysis of pancreatic sections revealed that T Ag-positive cells were detected in the islets of 1-year-old (Fig. 4, *G* and *H*) and 2-year-old (Fig. 4, *K* and *L*) RIP1-Tag4 mice. H&E staining of pancreatic tissue from these T Ag-immune mice showed that most islets were smaller than the islets found in naive 6-mo-old RIP1-Tag4 mice, although some hyperplastic islets were slightly larger (Fig. 4*F*). It should be noted that the number of islet cells positive for T Ag from immunized mice was generally reduced (Fig. 4, *G*, *K*, and *L*) compared with the number of T Ag-positive cells detected in tumors from unimmunized RIP1-Tag4 mice (Fig. 4*D*) unless a particular islet appeared hyperplastic (Fig. 4*H*). Hyperplastic islets were rare, however, in immunized mice (Fig. 3*D*). Thus, despite continuous expression of T Ag in the islets, large tumors did not develop in immunized mice. These results demonstrate that immunization at 35 days of age with T Ag epitope IV-only expressing cells prevented the development of tumors in RIP1-Tag4 mice. These data also suggest that T Ag-expressing islet cells may be susceptible to T_{CD8}-mediated elimination but that enough β cells persist to produce insulin over the lifespan of the mice.

Lifelong persistence of T Ag epitope IV-specific T_{CD8} in immunized RIP1-Tag4 mice

We next determined whether epitope IV-specific T_{CD8} induced by immunization with Tag IV-only cells at 35 days of age persist and remain functional in 2-year old RIP1-Tag4 survivor mice. Representative survivors were sacrificed to quantitate the epitope IV-specific T_{CD8} present in the spleens and pancreas by tetramer staining and to examine T cell function by in vivo cytotoxicity assay (Fig. 5*A*). This analysis revealed that epitope IV-specific T_{CD8} were present at detectable levels in the spleens (8.1% of total T_{CD8}) of long-term survivors and expanded dramatically (53% of total T_{CD8}) following in vitro culture (Fig. 5*A*; group 5). Analysis of epitope IV-specific in vivo cytotoxicity in the same animal showed that epitope IV-pulsed targets were completely eliminated. To determine whether these persisting epitope IV-specific T_{CD8} could expand following antigenic challenge in vivo, representative mice were immunized with B6/WT-19 cells. After nine days, epitope IV-specific T_{CD8} had expanded to 15% of T_{CD8} in the spleen (Fig. 5*A*; group 6), indicating that these persisting T_{CD8} remained sensitive to antigenic challenge. Epitope IV-pulsed target cells were also completely eliminated in these mice although no epitope I-specific lysis was detected, indicating that these RIP1-Tag4 mice remained fully tolerant to epitope I despite the lack of tumor progression. Thus, in contrast to the fate of epitope I-specific T_{CD8}, epitope IV-specific T_{CD8} primed from the endogenous repertoire by specific immunization persist long term in RIP1-Tag4 mice and remain functional even in aged mice.

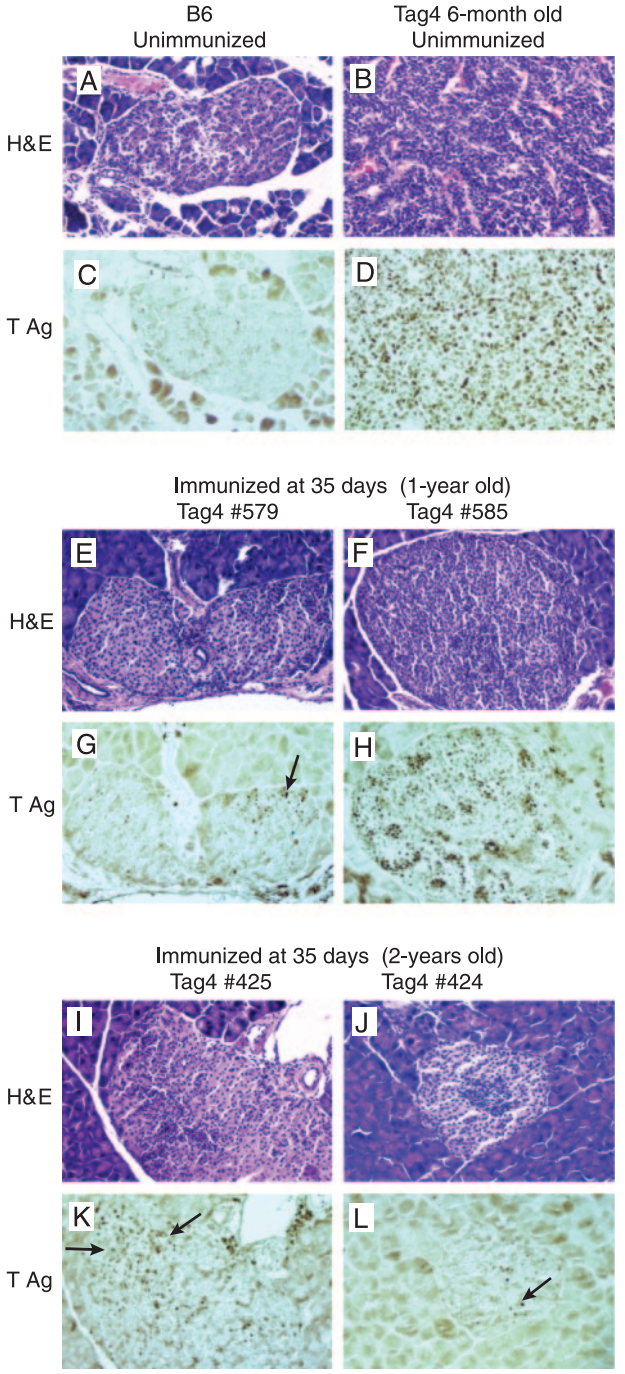

FIGURE 4. Immunized, long-term surviving RIP1-Tag4 mice maintain T Ag expression in the pancreas. Paraffin-embedded sections were prepared from pancreas of representative B6 mice (*A* and *C*), unimmunized 6-mo-old RIP1-Tag4 mice (*B* and *D*), 1-year-old RIP1-Tag4 mice immunized at 35 days of age with B6/15Bb cells (*E–H*; two individual mice are shown), and 2-year-old RIP1-Tag4 mice immunized at 35 days of age (*I—L*; two individual mice are shown). Parallel sections were stained by H&E (*A*, *B*, *E*, *F*, *I*, and *J*) and immunohistochemistry for T Ag (*C*, *D*, *G*, *H*, *K*, and *L*). All sections are representative of multiple fields for each mouse shown except *F* and *H*, which show a single neoplastic islet that was detected in a long-term survivor. Black arrows indicate T Ag-positive cells within the islets. Original magnification was ×200. Staining of nuclear T Ag appears brown.

For continuous inhibition of tumor growth to occur, epitope IV-specific T_{CD8} induced by immunization should be present in the

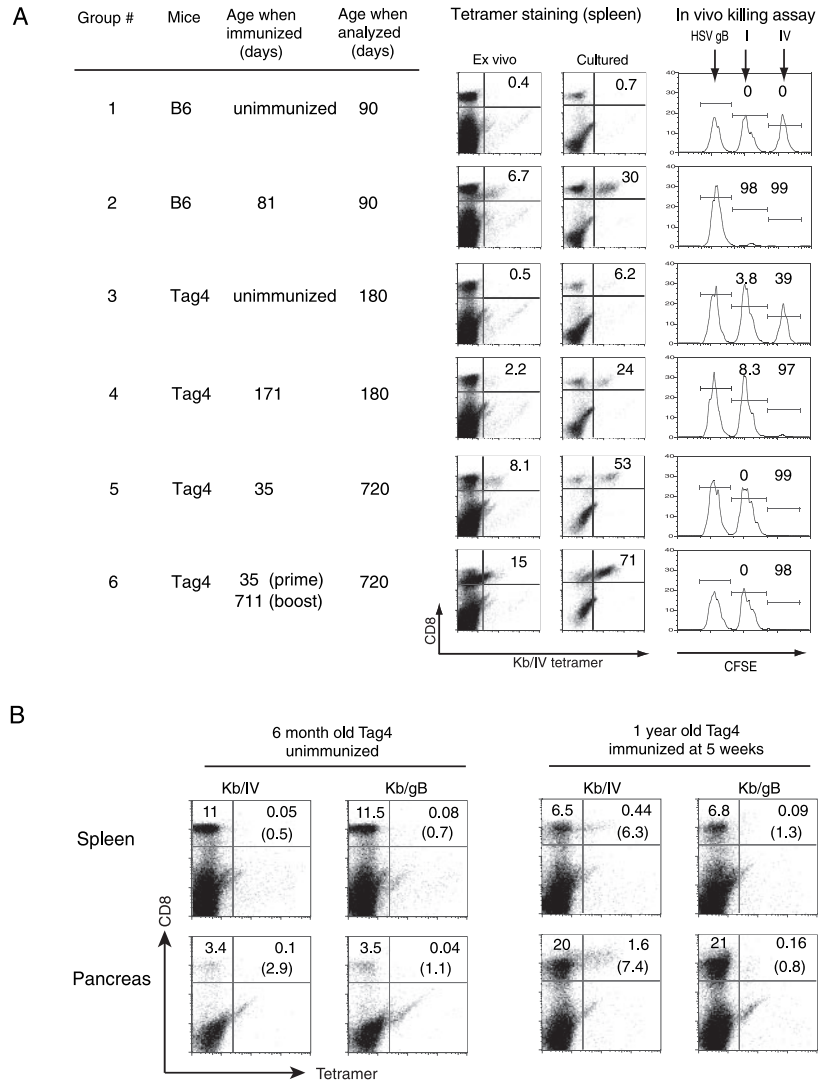

FIGURE 5. Epitope IV-specific T_{CD8} remain functional in RIP1-Tag4 mice despite continuous expression of T Ag. *A*, Spleen cells derived from RIP1-Tag4 (Tag4) mice and control B6 mice that were immunized with either B6/WT-19 cells (groups 2 and 4) or epitope IV-only T Ag-expressing B6/15Bb cells (groups 5 and 6) at the indicated ages were analyzed for the presence of epitope IV-specific T_{CD8} by staining with the Kb/IV tetramer ex vivo (*left panels*) and after in vitro culture with γ-irradiated B6/WT-19 cells (*middle panels*). Mice in group 6 received a booster immunization with B6/WT-19 cells 9 days before analysis. The values in the *upper right quadrant* indicate the percentage of epitope IV-specific T cells per total T_{CD8}. The effector functions of T Ag-specific T_{CD8} were determined using an in vivo cytotoxicity assay (*right panels*) against B6/SJL target cells incubated with peptide IV ($CFSE^{high}$), peptide I ($CFSE^{medium}$), and control peptide HSV gB ($CFSE^{low}$). Data are representative of those obtained for two to three mice per group. Values over each histogram represent the percentage of target cells eliminated in vivo. *B*, Pancreata from long-term surviving immunized RIP1-Tag4 mice contain epitope IV-specific T_{CD8}. Lymphocytes were isolated from pancreatic tissues and spleens of 6-mo-old untreated RIP1-Tag4 mice and 1-year-old RIP1-Tag4 mice immunized at 5 wk of age with B6/15Bb cells and stained with Kb/IV tetramer or control Kb/gB tetramer. Values indicate the percentage of total cells in each quadrant or the percentage of T_{CD8} that stained positive with each tetramer (values in brackets). Data are representative of three mice that showed similar results.

pancreas of long-term surviving RIP1-Tag4 mice. Thus, we analyzed by tetramer staining enzymatically digested pancreata from 1-year-old RIP1-Tag4 mice immunized with epitope IV-only T Ag expressing B6/15Bb cells at 35 days of age. The data in Fig. 5*B* demonstrate that 7.4% of T_{CD8} in the pancreas stained positively with the Kb/IV tetramer at this late time point. Similarly, a slightly lower number (6.3%) of Kb/IV tetramer-positive T_{CD8} were detected in the spleen. In contrast, lymphocytes isolated from the spleen and pancreas of a naive 6-mo-old RIP1-Tag4 mouse were devoid of epitope IV-specific T_{CD8}, indicating that T cell accumulation in the pancreas requires specific immunization. Taken together, our data clearly show that epitope IV-specific T_{CD8} persist in the spleens and pancreas of RIP1-Tag4 mice immunized at 35 days of age and remain functional throughout the lifetime of the mice without being significantly tolerized.

Epitope IV-specific T_{CD8} activated in tumor-bearing mice are not functionally compromised

One possible explanation for the inability of immunization against T Ag to induce control of advanced-stage tumors in RIP1-Tag4 mice is that the responding epitope IV-specific T_{CD8} in older mice are rendered anergic or that higher avidity T_{CD8} are deleted following recognition of self Ag (7, 15, 41–43). Thus, we determined whether RIP1-Tag4 mice immunized at 6 mo of age could eliminate target cells pulsed with epitope IV peptide. Epitope IV-specific T_{CD8} were recruited to 2.2% of T_{CD8} following immunization of 171-day-old RIP1-Tag4 mice with wild-type T Ag-expressing B6/WT-19 cells (Fig. 5*A*; group 4). In addition, epitope IV-pulsed targets were completely eliminated in the in vivo killing assay, whereas no significant lysis of epitope I-pulsed target cells was observed. In contrast, both epitope I-specific and IV-specific killing was detected in control B6 mice immunized with B6/WT-19 cells (Fig. 5*A*; group 2). We considered that because epitope IV-specific T_{CD8} persisted in 6-mo-old naive RIP1-Tag4 mice, some spontaneous priming against the T Ag in tumor-bearing RIP1-Tag4 mice might occur. Spleen cells from unimmunized 6-mo-old RIP1-Tag4 mice were analyzed directly by tetramer staining. The results in Fig. 5*A* (group 3) show that although epitope IV-specific T_{CD8} were not detected directly ex vivo by MHC tetramer analysis, a modest level of epitope IV-specific killing was detected (39%). Moreover, epitope IV-specific T_{CD8} were expanded after in vitro culture (6.2% of T_{CD8}), indicating that RIP1-Tag4 mice spontaneously develop low numbers of activated epitope IV-specific T_{CD8}, a finding consistent with those reported by Ye et al. (18) in which SV40-specific CTL activity was detected ex vivo. No epitope IV-specific T_{CD8} were detected following in vitro culture of splenocytes from naive B6 mice (Fig. 5*A*; group 1). Thus, the T_{CD8} triggered by immunization in tumor-bearing RIP1-Tag4 mice remain functional in vivo.

To determine whether the avidity of epitope IV-specific T_{CD8} was altered in tumor-bearing RIP1-Tag4 mice, we immunized groups of 2-mo-old and tumor-bearing 8.5-mo-old RIP1-Tag4 mice as well as nontransgenic B6 mice with B6/WT-19 cells expressing wild-type T Ag. After 10 days, spleen cells were harvested and cultured for 5 days in vitro with irradiated B6/WT-19 cells to expand the low number of cells that are detected in the older RIP1-Tag4 mice (Fig. 1). All mice in the 8.5-mo-old group had extensive tumor burden, whereas those in the 2-mo-old group lacked detectable tumors (data not shown). Cells in the responding lymphocyte cultures were tested for their ability to produce IFN-γ in response to titrated amounts of epitope IV peptide in an intracellular cytokine assay. The results demonstrate that T_{CD8} derived from tumor-bearing RIP1-Tag4 mice recognized epitope IV peptide as efficiently as those derived from 2-mo-old RIP1-Tag4 mice (Fig. 6). In addition, the avidity of epitope IV-specific T_{CD8} derived from RIP1-Tag4 mice did not vary from B6-derived T_{CD8}. These results indicate that the avidity and function of epitope IV-specific T_{CD8} does not change significantly as tumors progress in RIP1-Tag4 mice, although the frequency of T_{CD8} that can be recruited decreases with age (Fig. 1).

Discussion

Differences in the effect of tolerance toward two distinct T Ag epitopes

The overall goal of this study was to determine the extent to which peripheral tolerance affects the ability to recruit T_{CD8} specific for two immunodominant T Ag epitopes derived from a single oncogenic protein and whether control of spontaneous tumor growth correlates with the presence of one or both of these T_{CD8} populations. Our results demonstrate that tumor progression in RIP1-Tag4 mice can be controlled for the natural lifespan by targeting T_{CD8} specific for the single immunodominant K^b-restricted epitope IV only if mice are immunized at the time of initial oncogene expression. Control of tumor development is associated with the persistence of functional epitope IV-specific T_{CD8} in the peripheral lymphoid organs as well as in the pancreas. Despite the persistence of epitope IV-specific T_{CD8} in these mice, T_{CD8} to a second T Ag epitope, the Db-restricted epitope I, are deleted or anergized during the early stages of tumor development as a result of their interaction with the endogenous T Ag, even when the precursor frequency is dramatically increased by the addition of epitope I-specific TCR transgenic T cells. Thus, the kinetics of tolerance is epitope-dependent, even though both epitopes are derived from the same protein.

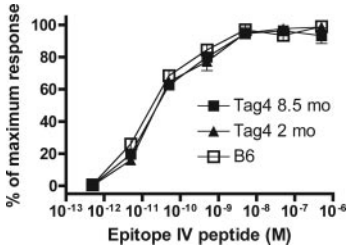

FIGURE 6. High avidity epitope IV-specific T_{CD8} survive in tumor-bearing RIP1-Tag4 mice. Groups of three RIP1-Tag4 mice at the indicated ages or control B6 mice were immunized i.p. with 3×10^7 B6/WT-19 cells. After 10 days, spleen cells were harvested and restimulated in vitro with γ-irradiated B6/WT-19 cells for 5 days. Responder cells were plated at 1×10^6 cells per well and incubated in the presence of the indicated dilutions of epitope IV peptide or a control peptide plus brefeldin A for 6 h at 37°C. Cells were stained for CD8 and intracellular IFN-γ, and the percentage of responding cells was calculated at each peptide dilution. Data are expressed as the percentage of the maximum response obtained for each culture. Data points represent three mice per group $\pm$ SE. The percentage of $CD8^+$ cells producing IFN-γ at the lowest peptide IV dilution was 8.8, 21.1, and 18.5% for 8.5-mo-old RIP1-Tag4, 2-mo-old RIP1-Tag4, and B6 mice, respectively. This experiment was performed twice with similar results.

The finding that complete T_{CD8} tolerance toward epitope I occurs despite only partial loss of responsiveness to epitope IV is consistent with a previous study using a different line of SV40 T Ag transgenic mice in which epitope I-specific T_{CD8} were shown to be more rapidly tolerized by the endogenous T Ag than epitope IV-specific T_{CD8}. Line 501 mice express T Ag from the α-amylase promoter, resulting in the formation of osteosarcomas around 1 year of age (44). Similar to RIP1-Tag4 mice, T_{CD8} specific for both T Ag epitopes are initially detected following immunization of young 501 mice (6), but these mice become tolerant to epitope I by 6 mo of age when high levels of T Ag expression are detected. Epitope IV-specific T_{CD8} were efficiently recruited in 501 mice at 6 mo of age, but unlike the present study these cells were eventually tolerized following the appearance of tumors. One possible mechanism to explain the early loss of epitope I-specific T_{CD8} in these two different T Ag transgenic mouse models is that a difference in the T_{CD8} precursor frequency specific for epitopes I and IV may lead to more rapid loss of epitope I responsiveness. This suggestion is based on the finding that epitope IV-specific T_{CD8} accumulate at higher levels than epitope I-specific T_{CD8} in both B6 and RIP1-Tag4 mice following immunization with wild-type T Ag. Whether the magnitude of the response can be attributed to differences in the naive precursor frequency or to other mechanisms that control immunodominance remains to be determined. Our experiments here tested this possibility by seeding in large numbers of TCR-I T cells to increase the naive precursor frequency as well as the frequency of responder cells following immunization. In both cases, the epitope I-specific T_{CD8} were lost rapidly from the RIP1-Tag4 hosts, suggesting that the differential tolerance observed is not explained simply by the presence of fewer epitope I-specific T_{CD8}. Rather, our data support a model in which T_{CD8} specific for epitope I may be more susceptible to peripheral tolerance than T_{CD8} specific for epitope IV. Some factors that have been shown to contribute to peripheral T cell tolerance include the level of Ag expression (45, 46), lack of $CD4^+$ T cell help (47), and the avidity of self-reactive T cells (7). Because both epitopes are derived from the same protein, it is unlikely that protein levels contribute toward this difference. Epitope I, however, is known to form highly stable complexes with Db (48), perhaps leading to prolonged high level presentation in vivo that results in more rapid deletion of the responding T cells. Whether endogenous T cells specific for epitopes I and IV from RIP1-Tag4 mice have comparable avidities is unknown.

A unique finding in this study is that a proportion of epitope IV-specific T_{CD8} survive the effects of peripheral tolerance in RIP1-Tag4 mice even after the appearance of tumors. This contrasts with other models of T Ag-induced spontaneous cancer in which epitope IV-specific T_{CD8} are eventually tolerized in mice with progressively growing tumors (6, 21). Epitope IV-specific T_{CD8} are apparently deleted from the repertoire of line 501 mice developing osteosarcomas, as they are undetectable following immunization of tumor-bearing animals but respond to immunization in age-matched littermates without tumors (6). This difference might be due to contrasting properties of osteosarcomas developing in 501 mice and insulinomas in RIP1-Tag4 mice. Tumors in 501 mice appear at a later age, grow invasively into surrounding tissues, and are metastatic (44). In contrast, tumors in RIP1-Tag4

mice do not infiltrate surrounding tissues and do not form metastases. In addition, T Ag is expressed in multiple tissues in 501 mice, whereas expression is localized to the pancreatic β cells in RIP1-Tag4 mice.

A different scenario is observed when a silent T Ag transgene, expressed from the β-actin/β-globin promoter, is activated and leads to sporadic tumor formation in various tissues (21). In this case, epitope IV-specific T_{CD8} appear to be rendered noncytolytic by the tumor environment, perhaps due to the presence of immunosuppressive cytokines such as TGF-β. This finding contrasts with our observation in which epitope IV-specific T_{CD8} remained cytotoxic even in mice with advanced-stage tumors. These differences are a reminder that unique tumor environments can differentially affect T_{CD8} responding to the same tumor-specific epitope and that the results cannot be generally applied to all tumor models.

Tumor progression in the presence of competent epitope IV-specific T_{CD8}

We found no evidence that tumor progression in naive RIP1-Tag4 mice altered the quality of epitope IV-specific T_{CD8}, although the number of responding cells is reduced 3-fold in tumor-bearing mice vs 5-wk old mice. Indeed, epitope IV-specific T_{CD8} generated in tumor-bearing mice acquired in vivo killing activity and the ability to produce IFN-γ and had a similar avidity for epitope IV peptide as those from normal B6 mice. Yet these T_{CD8} failed to significantly control tumor growth. A different result was obtained in mice coexpressing the lymphocytic choriomeningitis virus (LCMV) glycoprotein and T Ag from RIP in which immunization of tumor-bearing mice with LCMV was shown to provide a delay in tumor progression that waned following elimination of the virus (49). Similar to our findings, tumor progression continued despite the presence of persisting functional T_{CD8} targeting the immunodominant LCMV epitope. Our findings also contrast with those from some other tumor systems (50) in which tumor-infiltrating T_{CD8} were impaired in their ability to effectively destroy progressing tumors due to production of immunosuppressive cytokines (51, 52), inhibition by regulatory T cells (53–55), induction of apoptosis (56), or failure to infiltrate existing tumors (22). Whether T_{CD8} within the tumor stroma of RIP1-Tag4 mice are inhibited in situ remains to be determined.

Because epitope IV-specific T_{CD8} remain responsive and functional in RIP1-Tag4 mice with progressing tumors, escape from immune-mediated control of tumor progression may be related to changes in the tumor cells themselves. One such mechanism is the down-regulation of MHC class I expression, enabling tumor escape from immunosurveillance (57–59). Extensive analysis of MHC class I expression on islet cells in RIP1-Tag4 mice by Ye et al. (60) showed that normal islets in B6 and RIP1-Tag4 mice express MHC class I, whereas insulinomas from unimmunized RIP1-Tag4 mice contained a heterogeneous population of cells that were either negative or weakly positive for MHC class I expression. This observation suggests that heterogeneity of MHC class I expression on tumor cells could limit the efficiency of immunosurveillance in 6-mo-old RIP1-Tag4 mice by preventing the recognition of T Ag-expressing tumor cells.

The basis for complete protection in RIP1-Tag4 mice immunized before but not after tumor formation may lie in the steps that lead to T Ag transformation and tumorigenesis. T Ag-expressing cells undergo immortalization with the capacity to proliferate followed by acquisition of a tumorigenic phenotype (61). A brief interaction with activated functional T Ag-specific T_{CD8} can interfere with this process. Our previous studies (62) have shown that T Ag-transfected primary mouse embryo fibroblasts abort the transformation process upon a brief interaction with a T Ag-specific CTL clone. In this study we show that a portion of pancreatic islets of RIP1-Tag4 mice that were immunized at 35 days of age still contain cells positive for T Ag expression at 2 years of age, and most do not appear to be hyperplastic. The mechanism by which epitope IV-specific T_{CD8} suppress tumor progression in vivo could be mediated by the production of cytokines such as IFN-γ or TNF-α (63, 64). Alternatively, those cells expressing the highest levels of T Ag might be eliminated before transitioning to tumor cells. This mechanism requires sufficient levels of MHC class I expression to present T Ag epitopes to functional T_{CD8}, whereas escape variants arising because of low MHC class I expression would be expected to quickly advance to progressively growing tumors.

Lifelong tumor control without development of diabetes

Our results provide support for an earlier observation (18) that RIP1-Tag4 mice immunized with SV40 before endogenous T Ag expression led to long-term control of tumor progression (average lifespan of 622 days in the previous study). However, immunization with SV40 was ineffective after T Ag was expressed from the host's own β cells despite the persistence of SV40-specific CTL. Although the vehicle used for immunization (SV40 vs T Ag transformed cells) differs from that in the previous study, our findings here suggest that control of tumor progression following SV40 infection of young RIP1-Tag4 mice in the previous study was likely mediated by epitope IV-specific T_{CD8}. A similar result was obtained recently in the related RIP1-Tag5 line of mice following immunization with soluble T Ag in the presence of CpG-containing oligonucleotides (22), although no quantitative data on the T_{CD8} response was presented.

We found that long-term control of tumor development by epitope IV-specific T_{CD8} does not result in diabetes in RIP1-Tag4 mice, because sufficient numbers of T Ag-expressing β cells survive. This is despite the persistence of functionally competent epitope IV-specific T_{CD8}. Thus, an equilibrium is reached in which tumor progression is controlled but organ function is maintained, an ideal scenario for tumor immunotherapy. Our results contrast with those observed in mice expressing either the glycoprotein or NP from LCMV in β cells. In this case, virus infection resulted in T cell infiltration followed by diabetes due to destruction of the β cells (39, 40). Indeed, we found that T Ag-expressing cells persisted in the islets of long-term survivors. Whether continued β-islet cell survival in these mice is dependent on the level of T Ag expression is unknown. Defining the basis for the establishment of this equilibrium may yield new concepts for targeting immunotherapy against self-Ags.

A similar scenario has been observed in some transplantable tumor models in which immunization against a self-tumor Ag induces tumor regression without leading to autoimmunity (5, 65, 66). Thus, the number of insulin-secreting T Ag-positive β cells that remain produce enough insulin to prevent the development of diabetes. It should be pointed out that all β cells in RIP1-Tag4 transgenic mice are expected to express T Ag, although the expression levels may vary (32). In the RIP-hemagglutinin (HA) transgenic model, immunization with Flu also induces the onset of diabetes due to the activation of endogenous self-reactive T_{CD8} (42). In this model, however, RIP-HA mice must be immunized before 1 wk of age to induce diabetes, because high avidity HA-specific T_{CD8} are eliminated by peripheral tolerance at later time points (7). Preliminary experiments suggest that epitope IV-specific T_{CD8} recovered from long-term surviving RIP1-Tag4 mice have avidities similar to those derived from B6 mice. The possibility remains that the LCMV and Flu HA proteins are expressed

at higher levels in the pancreas than T Ag, such that β cells in these mice are more readily destroyed.

A recently published study argued against a role for immunosurveillance of spontaneous cancer (21). That suggestion may not be generalized to all immunogenic spontaneous models of cancer (67). Although the role of spontaneously primed T_{CD8} to control autochthonous tumors will continue to be debated, data presented here and by others (18, 21) argue that long-term immunosurveillance against spontaneous tumors can be effective if T cells are activated before the appearance of tumors or if the tumor-bearing host is preconditioned before immunotherapy (14, 31, 68). A key to effective T cell-mediated therapy is to target epitopes less likely to lead to peripheral tolerance following prolonged exposure to the endogenous Ag. In addition, understanding the mechanisms by which tumor-specific T cell responses can mediate control of tumor progression without disrupting organ function will be a major advance toward developing immunotherapeutic strategies.

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Disclosures

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References

1. Pardoll, D. 2003. Does the immune system see tumors as foreign or self? *Annu. Rev. Immunol.* 21: 807–839.
2. Theobald, M., J. Biggs, J. Hernandez, J. Lustgarten, C. Labadie, and L. A. Sherman. 1997. Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. *J. Exp. Med.* 185: 833–841.
3. Schell, T. D., L. M. Mylin, I. Georgoff, A. K. Teresky, A. J. Levine, and S. S. Tevethia. 1999. Cytotoxic T-lymphocyte epitope immunodominance in the control of choroid plexus tumors in simian virus 40 large T antigen transgenic mice. *J. Virol.* 73: 5981–5993.
4. Colella, T. A., T. N. Bullock, L. B. Russell, D. W. Mullins, W. W. Overwijk, C. J. Luckey, R. A. Pierce, N. P. Restifo, and V. H. Engelhard. 2000. Self-tolerance to the murine homologue of a tyrosinase-derived melanoma antigen: implications for tumor immunotherapy. *J. Exp. Med.* 191: 1221–1232.
5. Morgan, D. J., H. T. Kreuwel, S. Fleck, H. I. Levitsky, D. M. Pardoll, and L. A. Sherman. 1998. Activation of low avidity CTL specific for a self epitope results in tumor rejection but not autoimmunity. *J. Immunol.* 160: 643–651.
6. Schell, T. D., B. B. Knowles, and S. S. Tevethia. 2000. Sequential loss of cytotoxic T lymphocyte responses to simian virus 40 large T antigen epitopes in T antigen transgenic mice developing osteosarcomas. *Cancer Res.* 60: 3002–3012.
7. Nugent, C. T., D. J. Morgan, J. A. Biggs, A. Ko, I. M. Pilip, E. G. Pamer, and L. A. Sherman. 2000. Characterization of $CD8^+$ T lymphocytes that persist after peripheral tolerance to a self antigen expressed in the pancreas. *J. Immunol.* 164: 191–200.
8. Cordaro, T. A., K. E. de Visser, F. H. Tirion, T. N. Schumacher, and A. M. Kruisbeek. 2002. Can the low-avidity self-specific T cell repertoire be exploited for tumor rejection? *J. Immunol.* 168: 651–660.
9. Ganss, R., and D. Hanahan. 1998. Tumor microenvironment can restrict the effectiveness of activated antitumor lymphocytes. *Cancer Res.* 58: 4673–4681.
10. Prevost-Blondel, A., C. Zimmermann, C. Stemmer, P. Kulmburg, F. M. Rosenthal, and H. Pircher. 1998. Tumor-infiltrating lymphocytes exhibiting high ex vivo cytolytic activity fail to prevent murine melanoma tumor growth in vivo. *J. Immunol.* 161: 2187–2194.
11. Ochsenbein, A. F., S. Sierro, B. Odermatt, M. Pericin, U. Karrer, J. Hermans, S. Hemmi, H. Hengartner, and R. M. Zinkernagel. 2001. Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction. *Nature* 411: 1058–1064.
12. Minev, B. R., B. J. McFarland, P. J. Spiess, S. A. Rosenberg, and N. P. Restifo. 1994. Insertion signal sequence fused to minimal peptides elicits specific $CD8^+$ T cell responses and prolongs survival of thymoma-bearing mice. *Cancer Res.* 54: 4155–4161.
13. Nguyen, L. T., A. R. Elford, K. Murakami, K. M. Garza, S. P. Schoenberger, B. Odermatt, D. E. Speiser, and P. S. Ohashi. 2002. Tumor growth enhances cross-presentation leading to limited T cell activation without tolerance. *J. Exp. Med.* 195: 423–435.
14. Ganss, R., E. Ryschich, E. Klar, B. Arnold, and G. J. Hammerling. 2002. Combination of T-cell therapy and trigger of inflammation induces remodeling of the vasculature and tumor eradication. *Cancer Res.* 62: 1462–1470.
15. Staveley-O'Carroll, K., T. D. Schell, M. Jimenez, L. M. Mylin, M. J. Tevethia, S. P. Schoenberger, and S. S. Tevethia. 2003. In vivo ligation of CD40 enhances priming against the endogenous tumor antigen and promotes $CD8^+$ T cell effector function in SV40 T antigen transgenic mice. *J. Immunol.* 171: 697–707.
16. Lyman, M. A., S. Aung, J. A. Biggs, and L. A. Sherman. 2004. A spontaneously arising pancreatic tumor does not promote the differentiation of naive $CD8^+$ T lymphocytes into effector CTL. *J. Immunol.* 172: 6558–6567.
17. Lyman, M. A., C. T. Nugent, K. L. Marquardt, J. A. Biggs, E. G. Pamer, and L. A. Sherman. 2005. The fate of low affinity tumor-specific $CD8^+$ T cells in tumor-bearing mice. *J. Immunol.* 174: 2563–2572.
18. Ye, X., J. McCarrick, L. Jewett, and B. B. Knowles. 1994. Timely immunization subverts the development of peripheral nonresponsiveness and suppresses tumor development in simian virus 40 tumor antigen-transgenic mice. *Proc. Natl. Acad. Sci. USA* 91: 3916–3920.
19. Marton, I., S. E. Johnson, I. Damjanov, K. S. Currier, J. P. Sundberg, and B. B. Knowles. 2000. Expression and immune recognition of SV40 tag in transgenic mice that develop metastatic osteosarcomas. *Transgenic Res.* 9: 115–125.
20. Reilly, R. T., M. B. Gottlieb, A. M. Ercolini, J. P. Machiels, C. E. Kane, F. I. Okoye, W. J. Muller, K. H. Dixon, and E. M. Jaffee. 2000. HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. *Cancer Res.* 60: 3569–3576.
21. Willimsky, G., and T. Blankenstein. 2005. Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. *Nature* 437: 141–146.
22. Garbi, N., B. Arnold, S. Gordon, G. J. Hammerling, and R. Ganss. 2004. CpG motifs as proinflammatory factors render autochthonous tumors permissive for infiltration and destruction. *J. Immunol.* 172: 5861–5869.
23. Yewdell, J. W., and J. R. Bennink. 1992. Cell biology of antigen processing and presentation to major histocompatability complex molecule-restricted T lymphocytes. *Adv. Immunol.* 52: 1–123.
24. Boon, T., P. G. Coulie, B. J. Van den Eynde, and P. Van der Bruggen. 2005. Human T cell responses against melanoma. *Annu. Rev. Immunol.* 24: 175–208.
25. Yewdell, J. W., and J. R. Bennink. 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. *Annu. Rev. Immunol.* 17: 51–88.
26. Tevethia, S. S., and T. D. Schell. 2002. The immune response to SV40, JCV and BKV. In *Human Polyomaviruses: Molecular and Clinical Perspectives*, K. Khalili, and G. L. Stoner, eds. Wiley-Liss, New York, p. 585–610.
27. Mylin, L. M., T. D. Schell, D. Roberts, M. Epler, A. Boesteanu, E. J. Collins, J. A. Frelinger, S. Joyce, and S. S. Tevethia. 2000. Quantitation of $CD8^+$ T-lymphocyte responses to multiple epitopes from simian virus 40 (SV40) large T antigen in C57BL/6 mice immunized with SV40, SV40 T-antigen-transformed cells, or vaccinia virus recombinants expressing full-length T antigen or epitope minigenes. *J. Virol.* 74: 6922–6934.
28. Mylin, L. M., A. M. Deckhut, R. H. Bonneau, T. D. Kierstead, M. J. Tevethia, D. T. Simmons, and S. S. Tevethia. 1995. Cytotoxic T lymphocyte escape variants, induced mutations, and synthetic peptides define a dominant H-$2K^b$-restricted determinant in simian virus 40 tumor antigen. *Virology* 208: 159–172.
29. Tanaka, Y., R. W. Anderson, W. L. Maloy, and S. S. Tevethia. 1989. Localization of an immunorecessive epitope on SV40 T antigen by H-$2D^b$-restricted cytotoxic T-lymphocyte clones and a synthetic peptide. *Virology* 171: 205–213.
30. Deckhut, A. M., J. D. Lippolis, and S. S. Tevethia. 1992. Comparative analysis of core amino acid residues of H-$2D^b$-restricted cytotoxic T-lymphocyte recognition epitopes in simian virus 40 T antigen. *J. Virol.* 66: 440–447.
31. Schell, T. D., and S. S. Tevethia. 2001. Control of advanced choroid plexus tumors in SV40 T antigen transgenic mice following priming of donor $CD8^+$ T lymphocytes by the endogenous tumor antigen. *J. Immunol.* 167: 6947–6956.
32. Hanahan, D. 1985. Heritable formation of pancreatic β-cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* 315: 115–122.
33. Adams, T. E., S. Alpert, and D. Hanahan. 1987. Non-tolerance and autoantibodies to a transgenic self antigen expressed in pancreatic β cells. *Nature* 325: 223–228.
34. Pretell, J., R. S. Greenfield, and S. S. Tevethia. 1979. Biology of simian virus 40 (SV40) transplantation rejection antigen (TrAg). In vitro demonstration of SV40 TrAg in SV40 infected non-permissive mouse cells by the lymphocyte-mediated cytotoxicity assay. *Virology* 97: 32–41.
35. Bonneau, R. H., L. H. Salvucci, D. C. Johnson, and S. S. Tevethia. 1993. Epitope specificity of H-$2K^b$-restricted, HSV-1-, and HSV-2-cross-reactive cytotoxic T lymphocyte clones. *Virology* 195: 62–70.
36. Harlow, E., L. V. Crawford, D. C. Pim, and N. M. Williamson. 1981. Monoclonal antibodies specific for simian virus 40 tumor antigens. *J. Virol.* 39: 861–869.
37. Cavender, J. F., A. Conn, M. Epler, H. Lacko, and M. J. Tevethia. 1995. Simian virus 40 large T antigen contains two independent activities that cooperate with a *ras* oncogene to transform rat embryo fibroblasts. *J. Virol.* 69: 923–934.
38. Radvanyi, F., S. Christgau, S. Baekkeskov, C. Jolicoeur, and D. Hanahan. 1993. Pancreatic β cells cultured from individual preneoplastic foci in a multistage tumorigenesis pathway: a potentially general technique for isolating physiologically representative cell lines. *Mol. Cell. Biol.* 13: 4223–4232.
39. von Herrath, M. G., S. Guerder, H. Lewicki, R. A. Flavell, and M. B. A. Oldstone. 1995. Coexpression of B7-1 and viral ("self") transgenes in pancreatic β cells can break peripheral ignorance and lead to spontaneous autoimmune diabetes. *Immunity* 3: 727–738.
40. Ohashi, P. S., S. Oehen, K. Buerki, H. Pircher, C. T. Ohashi, B. Odermatt, B. Malissen, R. M. Zinkernagel, and H. Hengartner. 1991. Ablation of "tolerance" and induction of diabetes by virus infection in viral antigen transgenic mice. *Cell* 65: 305–317.
41. Kurts, C., H. Kosaka, F. R. Carbone, J. F. Miller, and W. R. Heath. 1997. Class I-restricted cross-presentation of exogenous self-antigens leads to deletion of autoreactive $CD8^+$ T cells. *J. Exp. Med.* 186: 239–245.

42. Morgan, D. J., C. Kurts, H. T. Kreuwel, K. L. Holst, W. R. Heath, and L. A. Sherman. 1999. Ontogeny of T cell tolerance to peripherally expressed antigens. *Proc. Natl. Acad. Sci. USA* 96: 3854–3858.
43. Sandberg, J. K., L. Franksson, J. Sundback, J. Michaelsson, M. Petersson, A. Achour, R. P. Wallin, N. E. Sherman, T. Bergman, H. Jornvall, et al. 2000. T cell tolerance based on avidity thresholds rather than complete deletion allows maintenance of maximal repertoire diversity. *J. Immunol.* 165: 25–33.
44. Knowles, B. B., J. McCarrick, N. Fox, D. Solter, and I. Damjanov. 1990. Osteosarcomas in transgenic mice expressing an α-amylase-SV40 T-antigen hybrid gene. *Am. J. Pathol.* 137: 259–262.
45. Kurts, C., R. M. Sutherland, G. Davey, M. Li, A. M. Lew, E. Blanas, F. R. Carbone, J. F. Miller, and W. R. Heath. 1999. CD8 T cell ignorance or tolerance to islet antigens depends on antigen dose. *Proc. Natl. Acad. Sci. USA* 96: 12703–12707.
46. Morgan, D. J., H. T. Kreuwel, and L. A. Sherman. 1999. Antigen concentration and precursor frequency determine the rate of $CD8^+$ T cell tolerance to peripherally expressed antigens. *J. Immunol.* 163: 723–727.
47. Kurts, C., F. R. Carbone, M. Barnden, E. Blanas, J. Allison, W. R. Heath, and J. F. Miller. 1997. $CD4^+$ T cell help impairs $CD8^+$ T cell deletion induced by cross-presentation of self-antigens and favors autoimmunity. *J. Exp. Med.* 186: 2057–2062.
48. Fu, T.-M., L. M. Mylin, T. D. Schell, I. Bacik, G. Russ, J. W. Yewdell, J. R. Bennink, and S. S. Tevethia. 1998. An endoplasmic reticulum-targeting signal sequence enhances the immunogenicity of an immunorecessive simian virus 40 large T antigen cytotoxic T-lymphocyte epitope. *J. Virol.* 72: 1469–1481.
49. Speiser, D. E., R. Miranda, A. Zakarian, M. F. Bachmann, K. McKall-Faienza, B. Odermatt, D. Hanahan, R. M. Zinkernagel, and P. S. Ohashi. 1997. Self antigens expressed by solid tumors do not efficiently stimulate naive or activated T cells: implications for immunotherapy. *J. Exp. Med.* 186: 645–653.
50. Whiteside, T. L. 1998. Immune cells in the tumor microenvironment. Mechanisms responsible for functional and signaling defects. *Adv. Exp. Med. Biol.* 451: 167–171.
51. Oyama, T., S. Ran, T. Ishida, S. Nadaf, L. Kerr, D. P. Carbone, and D. I. Gabrilovich. 1998. Vascular endothelial growth factor affects dendritic cell maturation through the inhibition of nuclear factor-κB activation in hemopoietic progenitor cells. *J. Immunol.* 160: 1224–1232.
52. Sharma, S., M. Stolina, Y. Lin, B. Gardner, P. W. Miller, M. Kronenberg, and S. M. Dubinett. 1999. T cell-derived IL-10 promotes lung cancer growth by suppressing both T cell and APC function. *J. Immunol.* 163: 5020–5028.
53. Gorelik, L., and R. A. Flavell. 2001. Immune-mediated eradication of tumors through the blockade of transforming growth factor-β signaling in T cells. *Nat. Med.* 7: 1118–1122.
54. Chen, M. L., M. J. Pittet, L. Gorelik, R. A. Flavell, R. Weissleder, H. von Boehmer, and K. Khazaie. 2005. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-β signals in vivo. *Proc. Natl. Acad. Sci. USA* 102: 419–424.
55. Wang, H. Y., D. A. Lee, G. Peng, Z. Guo, Y. Li, Y. Kiniwa, E. M. Shevach, and R. F. Wang. 2004. Tumor-specific human $CD4^+$ regulatory T cells and their ligands: implications for immunotherapy. *Immunity* 20: 107–118.
56. Whiteside, T. L. 2002. Tumor-induced death of immune cells: its mechanisms and consequences. *Semin. Cancer Biol.* 12: 43–50.
57. Ferrone, S., and F. M. Marincola. 1995. Loss of HLA class I antigens by melanoma cells: molecular mechanisms, functional significance and clinical relevance. *Immunol. Today* 16: 487–494.
58. Restifo, N. P., Y. Kawakami, F. Marincola, P. Shamamian, A. Taggarse, F. Esquivel, and S. A. Rosenberg. 1993. Molecular mechanisms used by tumors to escape immune recognition: immunogene therapy and the cell biology of major histocompatibility complex class I. *J. Immunother.* 14: 182–190.
59. Cromme, F. V., J. Airey, M. T. Heemels, H. L. Ploegh, P. J. Keating, P. L. Stern, C. J. Meijer, and J. M. Walboomers. 1994. Loss of transporter protein, encoded by the TAP-1 gene, is highly correlated with loss of HLA expression in cervical carcinomas. *J. Exp. Med.* 179: 335–340.
60. Ye, X., A. Kralli, R. Ge, R. P. Ricciardi, and B. B. Knowles. 1994. Down-regulation of MHC class I antigen in insulinoma cells controlled by the R1 element of the H-2 enhancer. *Oncogene* 9: 1195–1204.
61. Ahuja, D., M. T. Saenz-Robles, and J. M. Pipas. 2005. SV40 large T antigen targets multiple cellular pathways to elicit cellular transformation. *Oncogene* 24: 7729–7745.
62. Karjalainen, H. E., M. J. Tevethia, and S. S. Tevethia. 1985. Abrogation of simian virus 40 DNA-mediated transformation of primary C57BL/6 mouse embryo fibroblasts by exposure to a simian virus 40-specific cytotoxic T-lymphocyte clone. *J. Virol.* 56: 373–377.
63. Guidotti, L. G., and F. V. Chisari. 2001. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu. Rev. Immunol.* 19: 65–91.
64. Dunn, G. P., L. J. Old, and R. D. Schreiber. 2004. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* 21: 137–148.
65. Hodge, J. W., D. W. Grosenbach, W. M. Aarts, D. J. Poole, and J. Schlom. 2003. Vaccine therapy of established tumors in the absence of autoimmunity. *Clin. Cancer Res.* 9: 1837–1849.
66. Bronte, V., E. Apolloni, R. Ronca, P. Zamboni, W. W. Overwijk, D. R. Surman, N. P. Restifo, and P. Zanovello. 2000. Genetic vaccination with "self" tyrosinase-related protein 2 causes melanoma eradication but not vitiligo. *Cancer Res.* 60: 253–258.
67. Shankaran, V., H. Ikeda, A. T. Bruce, J. M. White, P. E. Swanson, L. J. Old, and R. D. Schreiber. 2001. IFN-γ and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 410: 1107–1111.
68. Dudley, M. E., J. R. Wunderlich, P. F. Robbins, J. C. Yang, P. Hwu, D. J. Schwartzentruber, S. L. Topalian, R. Sherry, N. P. Restifo, A. M. Hubicki, et al. 2002. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 298: 850–854.