

Tumor Growth Enhances Cross-Presentation Leading to Limited T Cell Activation without Tolerance

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Abstract

Using a tumor model of spontaneously arising insulinomas expressing a defined tumor-associated antigen, we investigated whether tumor growth promotes cross-presentation and tolerance of tumor-specific T cells. We found that an advanced tumor burden enhanced cross-presentation of tumor-associated antigens to high avidity tumor-specific T cells, inducing T cell proliferation and limited effector function *in vivo*. However, contrary to other models, tumor-specific T cells were not tolerized despite a high tumor burden. In fact, in tumor-bearing mice, persistence and responsiveness of adoptively transferred tumor-specific T cells were enhanced. Accordingly, a potent T cell-mediated antitumor response could be elicited by intravenous administration of tumor-derived peptide and agonistic anti-CD40 antibody or viral immunization and reimmunization. Thus, in this model, tumor growth promotes activation of high avidity tumor-specific T cells instead of tolerance. Therefore, the host remains responsive to T cell immunotherapy.

Key words: neoplasms • immunotherapy • cytotoxic T lymphocytes • immune tolerance • CD40

Introduction

The identification of tumor-associated antigens (TAAs)* has led to the development of therapeutic strategies aimed at activating host T cells to generate antitumor immune responses (1–3). Successful T cell immunotherapy depends on the presence of functional T cells specific for TAAs. Tumor-specific peripheral T cells in tumor-bearing hosts appear to have different fates, depending on the particular tumor model. A large number of mouse models and human studies have reported that tumor-specific T cells become tolerized in tumor-bearing hosts (4–15).

Tumor-induced tolerance induction may occur by a variety of mechanisms, such as clonal deletion or anergy (16). The induction of tolerance may occur after recognition of TAAs directly on tumor cells. For example, a lack of costimulatory molecules on tumor cells may render cognate T cells unable to respond to the TAAs (17). In the case of T cell tolerance to tumors located in tissues not normally surveyed by T cells, tolerance may be induced by cross-presentation of TAAs, where tumor-derived peptides are presented to T cells in the local draining LN by bone marrow-derived APCs (18, 19).

Although the factors influencing cross-presentation have yet to be fully defined, there is evidence that ligands present at a high concentration and those released by cellular destruction are most efficiently cross-presented (12, 20–23). Cross-presentation of self-antigen can mediate T cell tolerance in transgenic models (24, 25). Thus, in the case of a high tumor burden, where there are large numbers of tumor

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*Abbreviations used in this paper: LCMV-GP, lymphocytic choriomeningitis virus glycoprotein; NDLN, nondraining LN; PDLN, pancreatic draining LN; TAA, tumor-associated antigen.

cells and spontaneous tumor cell death, cross-presentation of TAAs may lead to tolerance of tumor-specific T cells.

To investigate whether tumor growth enhances cross-presentation of TAAs and tolerance of tumor-specific T cells, we used a model of naturally arising pancreatic cancer. RIP-Tag2 transgenic mice express the SV40 large T antigen (Tag) under the control of the rat insulin promoter (RIP; reference 26). Expression of Tag in pancreatic islet β cells contributes to β cell hyperplasia and development of solid, vascularized insulinomas at 10–12 wk of age, largely due to Tag-mediated inactivation of the Rb and p53 tumor suppressors (27, 28). Insulin secretion by these metabolically active tumor cells leads to progressive hypoglycemia, and untreated mice generally succumb to the insulinomas between 3–4 mo of age. Expression of Tag in this transgenic line also occurs during early embryogenesis and in the thymus, and thus RIP-Tag2 mice are immunologically tolerant to Tag (29, 30). To introduce a defined TAA, we generated a double transgenic model by breeding RIP-Tag2 mice with RIP-GP mice which express a glycoprotein from the lymphocytic choriomeningitis virus (LCMV-GP) also in pancreatic islet β cells (31, 32). For some experiments, we then crossed RIP(GP \times Tag2) double transgenic mice to the P14 TCR transgenic line, where TCR transgenic T cells express V α 2 and V β 8.1 chains that recognize the GP33 peptide from LCMV-GP in the context of H-2D^b (33). Thus, the generation of P14/RIP(GP \times Tag2) triple transgenic mice provided a model to directly examine the fate of endogenous tumor-specific T cells in the presence of advanced spontaneously arising tumors expressing a defined TAA.

We first investigated whether tumor-specific T cells became antigen-experienced in our model. We found that T cell activation markers were upregulated on GP-specific T cells in a manner dependent on presentation of TAAs by bone marrow-derived cells. Studies using P14/RIP(GP \times Tag2) triple transgenic mice demonstrated that tumor growth correlated with enhanced activation of tumor-specific T cells. We then determined whether recognition of TAA resulted in tolerance of tumor-specific T cells and assessed the effectiveness of T cell immunotherapeutic strategies in this model.

Materials and Methods

Mice and Tumor Monitoring. C57Bl/6 mice (H-2^b) were purchased from The Jackson Laboratory. RIP-Tag2 (originally described as RIP1-Tag2; reference 26), RIP-GP (31), and P14 TCR (33) transgenic lines were interbred to yield RIP(GP \times Tag2), P14/RIP-GP, P14/RIP-Tag2 double transgenic, and P14/RIP(GP \times Tag2) triple transgenic mice. P14/bm13 mice were generated as described previously (34). Genotyping for the P14 TCR transgene was performed by flow cytometric analysis using anti-V α 2 Ab (BD PharMingen). Genotyping for the RIP-Tag2 and RIP-GP transgenes was performed by PCR as described previously (32). Tumor progression was monitored by blood glucose measurements once every week from 8 wk of age onwards, and then 2–3 times per week after immunization. All experiments were performed using mice with blood glucose lev-

els at or below 5 mM, at which time they have solid tumors (26). The experimental endpoint occurred when blood glucose reached 2 mM. Blood glucose levels were measured using Chemstrips (Roche Laboratories) and Accu-Chek III glucometers (Boehringer Mannheim).

Flow Cytometric Analyses. Pancreatic draining LNs (PDLNs), pooled nondraining peripheral lymph nodes (inguinal, axillary, and cervical), and spleens were harvested and single cell suspensions prepared. Lymphocytes were stained with antibodies recognizing V α 2, V β 8.1/V β 8.2, CD8 α , CD44, CD69 (BD PharMingen), and detection of biotin-conjugated antibodies performed using streptavidin-conjugated red-670 (GIBCO BRL). For tetramer staining, spleen cells were incubated with CD8 α -FITC and H-2D^b/GP33-PE tetramers for 1 h on ice, at the indicated dilution factors, and then washed three times. Tetramers were provided by the tetramer core facility, National Institute of Allergy and Infectious Diseases, National Institutes of Health. Stained cells were fixed in 2% paraformaldehyde/PBS. Live events were collected based on forward and side scatter profiles on a FACScanTM flow cytometer (Becton Dickinson) and analyzed using CELLQuestTM software (Becton Dickinson).

Bone Marrow Chimeras. P14 and P14/bm13 donor mice were injected with CD4 (YTS191) and CD8 (YTS169) depleting antibodies intravenously 3 d and 1 d before bone marrow transfer. 10⁷ bone marrow cells were transferred intravenously into irradiated (9 Grays) sex-matched recipients, and recipients were killed for analysis 6–13 wk after reconstitution.

Adoptive Transfer of P14 TCR Transgenic Cells. Single cell suspensions were prepared from spleens of P14 TCR transgenic mice, and CD8⁺ T cells were purified using anti-CD8 α -coated beads (Miltenyi Biotech) and a magnetic activated cell sorter (Miltenyi Biotech). For adoptive transfer of unlabeled cells, 5 \times 10⁶ P14 T cells were transferred intravenously into unirradiated hosts, and hosts were killed for analysis 1, 2.5, and 6 wk after transfer. For adoptive transfer of CFSE-labeled cells, the purified cells were then labeled with CFSE (5-[and-6]-carboxyfluorescein diacetate, succinimidyl ester); after washing once in serum-free RPMI 1640 (GIBCO BRL), cells were resuspended in serum-free media at 10⁷ cells per 200 μ l containing 5 μ M CFSE (Molecular Probes). After incubation for 10 min at 37°C in an atmosphere containing 5% CO₂, cells were washed in RPMI 1640 containing 10% FCS (Sigma-Aldrich) and prepared for adoptive transfer. 5–10 \times 10⁶ CD8-purified, CFSE-labeled cells were transferred intravenously into nonirradiated recipients, and 72 h later, organs were harvested and stained for flow cytometric analysis.

Proliferation Assays. P14 TCR transgenic responder LN cells were cultured with an equivalent number of C57Bl/6 spleen cells and 10⁻⁶ M GP33 peptide (KAVYNFATM) or nonstimulatory AV peptide (SGPSNTPPEI; reference 35). To assay PDLN cells, 5 \times 10⁴ responders per well were cultured in round-bottomed 96-well plates. To assay pooled nondraining LN (NDLN) cells, 10⁵ responders per well were cultured in flat-bottomed 96-well plates. After 48 h, cultures were pulsed with 1 μ Ci/well [³H]thymidine (NEN Dupont) overnight and harvested onto glass fiber filters. [³H]thymidine incorporation was measured using a scintillation counter (Top-count; Canberra Packard).

CTL Assays. Mice were immunized with 2,000 PFU LCMV Armstrong intravenously. 8 d later, single cell suspensions of PDLN cells, NDLN cells, and splenocytes were prepared as effectors. Effectors from NDLN or spleens were plated at 6 \times 10⁵ cells per well, while PDLN cells were plated at 3 \times 10⁵ cells per well (due to limited cell numbers available from draining LNs) and 6 threefold serial dilutions performed. EL-4 target cells were

prepared by incubation with 500 $\mu\text{Ci/ml}$ ^{51}Cr (NEN Dupont) and 10^{-6} M MB6 (KAVVNIATM) or GP33 or nonstimulatory peptide AV (described above) for 2 h at 37°C . Targets were washed three times and plated at 10^4 cells per well with NDLN or spleen effectors, and at 5,000 cells per well with PDLN effectors in 96-well round-bottomed plates. After spinning at 1,000 rpm for 30 s, plates were incubated for 5 h at 37°C , and then 70 μl of supernatant was counted for 60 s using a Wallac Wizard γ counter (Perkin Elmer). Maximal release was induced by adding 1 M HCl to targets. Percentage of specific lysis was calculated as $(\text{cpm sample release} - \text{cpm spontaneous release}) / (\text{cpm maximal release} - \text{cpm spontaneous release}) \times 100$.

Immunotherapeutic Strategies. For peptide and antibody treatment, mice were treated with 5 μg of GP33 peptide in HBSS intravenously followed 2 d later with 100 μg of agonistic anti-CD40 mAb (FGK45; purified rat anti-mouse) (36) intravenously or 100 μg of rat polyclonal γ -globulin isotype-matched control (Bio/Can Scientific). For virus immunization, mice were treated with 2,000 PFU of LCMV Armstrong intravenously (provided by R.M. Zinkernagel, Institute for Experimental Immunology, Zürich, Switzerland), or 10^6 PFU of vacc-G2 intravenously (37) (provided by David Bishop, Oxford University, Oxford, UK), or 2,000 PFU of LCMV Armstrong intravenously followed by 10^6 PFU of vacc-G2 intravenously upon relapse of hypoglycemia. Blood glucose levels were monitored as described above.

Immunohistochemistry. Freshly removed pancreata were immersed in O.C.T. and snap frozen in liquid nitrogen. For the staining of cell differentiation markers, frozen tissue sections of 5- μm thickness were cut in a cryostat, placed on siliconized glass slides, air dried, fixed in acetone for 10 min, and stored at -70°C . Rehydrated tissue sections were incubated with primary rat mAbs against MHC class I (H-2K^b_{ds}, M1/42) and CD8 (YTS 169). Primary antibodies were revealed by sequential incubation with goat antibodies against rat Igs, followed by alkaline phosphatase labeled donkey antibodies against goat Igs (Jackson ImmunoResearch Laboratories). Dilutions of anti-Ig reagents were made in Tris buffered saline (TBS) containing 5% normal mouse serum. Alkaline phosphatase was visualized using naphthol AS-BI phosphate and new fuchsin as substrate, yielding a red color reaction product. Endogenous alkaline phosphatase was blocked by levanisole. Color reactions were performed at RT for 15 min with reagents from Sigma-Aldrich. Sections were counterstained with hemalum and coverslips mounted with glycerol and gelatin.

Results

Hypoglycemia Correlates with an Increased Tumor Burden in RIP-Tag2 Mice. In the RIP-Tag2 model, an increase in islet cell mass correlates with an increase in insulin production and a corresponding decrease in blood glucose levels. Animals were considered to have a significant tumor burden when blood glucose reach 5 mM. To quantitate the increase in islet cell mass, histological sections of pancreata from hypoglycemic RIP-Tag2 mice were compared with age-matched transgene-negative mice (Table I). Measurements of islets from random sections demonstrated a significant increase in the diameter of the islets in hypoglycemic RIP-Tag2 pancreata.

P14 T Cells Have High Avidity for LCMV-GP33. The prevailing belief is that tolerance induction generally occurs to self-antigen-specific T cells that are high avidity (2, 38–

41). Therefore, we wanted to determine whether the P14 transgenic tumor-specific T cells in our model represent a low or high avidity population. Using H-D^b/GP33 tetramers, we measured the mean fluorescence intensity of LCMV-specific T cells stained with different concentrations of tetramer, which reflects the relative TCR avidity (42, 43). A comparison of the curves for CD8⁺ T cells from P14 TCR transgenic mice versus the endogenous, LCMV-GP33-specific repertoire in nonTCR transgenic mice revealed that the P14 TCR represents a receptor with high avidity for LCMV-GP33. Surprisingly, the avidity is slightly higher than the endogenous C57Bl/6 repertoire expanded by LCMV (Fig. 1).

Enhanced Upregulation of CD44 on Tumor-specific T Cells in Tumor-bearing Mice. To determine whether tumor growth leads to increased stimulation of tumor-specific T cells, activation markers on P14 T cells were examined in a variety of transgenic mice. CD44 expression levels on P14 T cells from the PDLNs of P14/RIP(GP \times Tag2) triple transgenic mice with blood glucose levels <5 mM were compared with T cells from P14/RIP-GP mice. CD44 was upregulated on P14 T cells in the PDLNs of tumor-free P14/RIP-GP mice compared with control P14 or P14/RIP-Tag2 mice (Fig. 2 A and B). In tumor-bearing P14/RIP(GP \times Tag2) triple transgenic mice, CD44 expression on P14 T cells in the PDLNs was further upregulated compared with P14/RIP-GP mice (Fig. 2 A and B). P14 T cells isolated from NDLNs or spleens of P14/RIP-GP or P14/RIP(GP \times Tag2) mice did not show any upregulation of CD44 (Fig. 2 B), indicating that presentation of TAAs occurred only locally and not systemically. It remains possible that CD44 upregulation is caused by metabolic activation of the islet cells due to Tag expression. This analysis demonstrates that an increased tumor burden results in an increase in the proportion of T cells that encounter antigen.

Table I. Tumor Size in Hypoglycemic RIP-Tag2⁺ Mice

RIP-Tag2 ⁺	RIP-Tag2 ⁻
0.6	0.1
0.3	0.1
0.7	0.1
0.5	0.2
0.6	0.1
1.9	0.1
1.3	0.1
4.8	

Histological sections from pancreata of naive RIP-Tag2⁺ mice taken upon onset of blood glucose levels at or below 5 mM, or age-matched transgene-negative controls, were stained with H&E. Diameters of islets were measured on random sections of pancreata from 3 to 4 mice per group using vernier gradations on a light microscope. Data are diameters of normal islets (for transgene-negative samples) and hyperplastic or encapsulated islets (for RIP-Tag2⁺ samples), and are expressed in millimeters.

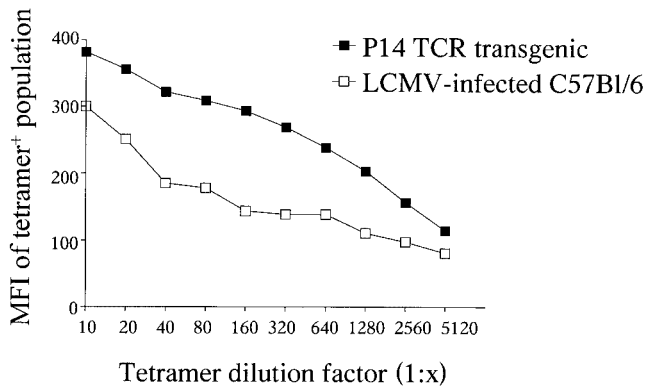


Figure 1. P14 TCR transgenic T cells have high avidity for LCMV-GP33. Splenocytes from naive P14 transgenic mice (black symbols) and C57Bl/6 mice 8 d after infection with 2,000 PFU LCMV Armstrong (white symbols) were stained with anti-CD8 α Ab and the indicated dilutions of H-2D^b/GP33 tetramer. Mean fluorescence intensities (MFI) of CD8⁺ T cells representative of 3–4 mice per group from two independent experiments are shown.

The LCMV-GP Is Cross-Presented in the PDLNs. To evaluate whether the mechanism by which tumor-specific T cells encounter the LCMV-GP was direct recognition of antigen presented by β cells or cross-presentation by bone marrow-derived cells, bone marrow chimeras were generated using H-2D^{bm13} bone marrow. H-2D^{bm13} encodes an altered H-2^b molecule that has three amino acid substitutions in the $\alpha 2$ peptide-binding domain (44). As a result, the LCMV-GP33 is no longer efficiently presented by H-2D^{bm13} (34). We generated chimeras by reconstituting RIP-GP, RIP-Tag2, and RIP(GP \times Tag2) mice with bone marrow from P14/H-2D^{bm13} (P14/bm13) mice or control P14/H-2D^b (P14/b) mice with wild-type H-2D^b molecules. Recognition of LCMV-GP by P14 transgenic T cells was assessed by staining for activation markers. Lethally irradiated RIP-GP and RIP(GP \times Tag2) recipients reconstituted with bone marrow cells from P14/b mice exhibited upregulation of CD69 (Fig. 3) and CD44 (data not shown) on P14 T cells specifically in the PDLNs. In contrast, P14 T cells from P14/bm13 \rightarrow RIP-GP or RIP(GP \times Tag2) chimeras, where GP33 could not be presented by bone marrow-derived cells, did not exhibit upregulation of CD69 (Fig. 3) or CD44 (data not shown). P14 T cells from control P14/b \rightarrow RIP-Tag2 and P14/bm13 \rightarrow RIP-Tag2 chimeras did not upregulate activation markers (data not shown), due to the lack of the LCMV-GP33 antigen. These observations indicate that tumor-specific T cells encounter cognate TAAs via cross-presentation in this model. This degree of cross-presentation was unable to induce significant tolerance of LCMV-GP-specific T cells, either in a single transgenic RIP-GP model or double transgenic P14/RIP-GP model that expresses a high proportion of LCMV-GP-specific T cells (31).

Spontaneous Induction of Proliferation and Effector Function of Tumor-specific T Cells. To evaluate whether the recognition of TAAs could induce proliferation of tumor-specific

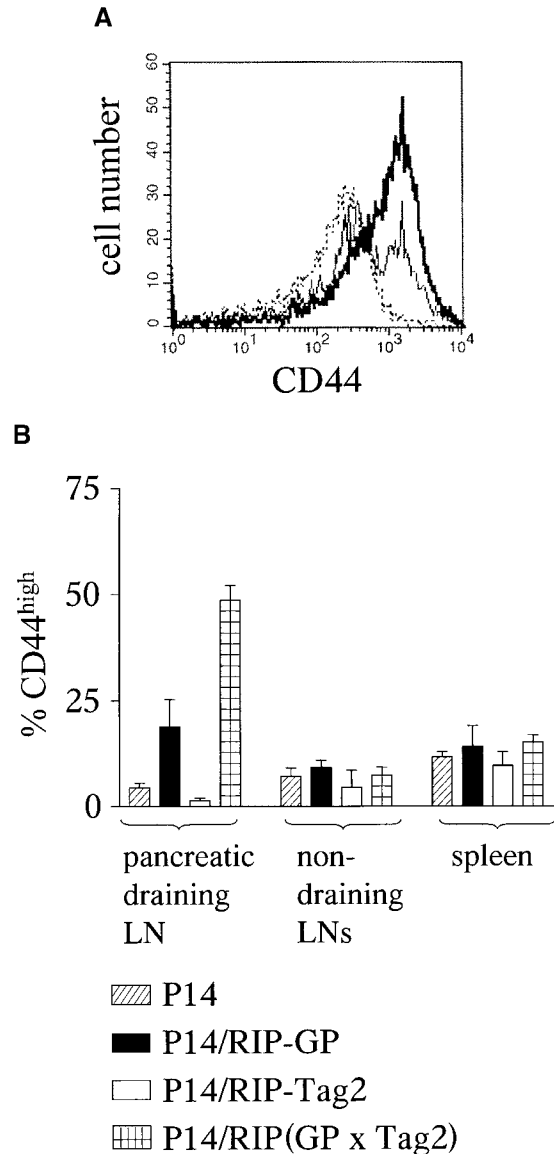


Figure 2. Enhanced upregulation of CD44 on tumor-specific T cells in the PDLNs. Lymphocytes from the PDLNs, NDLNs, and spleens of P14, P14/RIP-GP, and tumor-bearing P14/RIP-Tag2 and P14/RIP(GP-Tag2) mice were gated on V $\alpha 2$ ⁺ cells, and the percentage of CD44^{high} was determined. (A) Representative CD44 profiles of V $\alpha 2$ ⁺ PDLN cells are shown from P14 (dashed line), P14/RIP-GP (thin line), and P14/RIP(GP \times Tag2) (thick line) mice. (B) Data shown are the mean and standard error of 3–7 mice per genotype.

T cells, CD8⁺ T cells from naive P14 single transgenic mice were labeled with CFSE and adoptively transferred into RIP-GP, RIP(GP \times Tag2), and transgene-negative hosts (Fig. 4 A and B). 72 h after transfer, lymphocytes from PDLNs (Fig. 4 A), NDLNs (Fig. 4 B), and spleens (data not shown) were isolated from the hosts and CFSE profiles were evaluated by flow cytometric analysis. Cell division was induced in tumor-specific T cells recovered from PDLNs of RIP(GP \times Tag2) hosts (Fig. 4 A). No division was detected in transferred T cells recovered from NDLNs of RIP(GP \times Tag2) hosts (Fig. 4 B). In contrast to

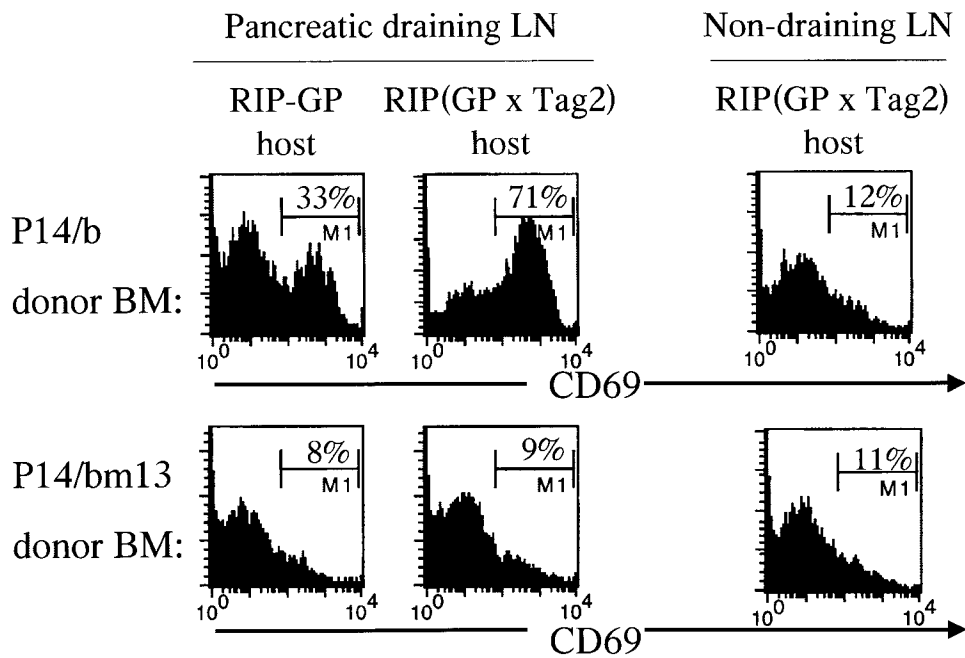


Figure 3. The LCMV-GP is cross-presented in the PDLNs. Bone-marrow cells from P14/b (top) or P14/bm13 (bottom) mice were transferred into lethally irradiated RIP-GP and RIP(GP × Tag2) recipients. Representative CD69 profiles (gated on Vα2⁺CD8⁺ cells) are shown for PDLNs and NDNLs from recipients 6 wk after transfer. Results are representative of 2–9 mice per group from three independent experiments.

the vigorous proliferation observed in RIP(GP × Tag2) hosts, weak proliferation of tumor-specific T cells was induced in RIP-GP hosts (Fig. 4 A). Thus, the presence of a tumor enhanced the quantity and/or quality of cross-presentation of self-antigen, resulting in enhanced proliferation of tumor-specific T cells.

To evaluate whether recognition of TAAs resulted in the generation of effector function in vivo, we compared the mean age of onset of hypoglycemia (≤ 5 mM blood glucose). We reasoned that any delay in the onset of hypoglycemia observed in naive P14/RIP(GP × Tag2) mice would reflect “spontaneous” activation of tumor-specific T cells and generation of a cytolytic antitumor response. RIP-Tag2, RIP(GP × Tag2), and P14/RIP-Tag2 mice all reached a blood glucose level ≤ 5 mM at a mean age of ~ 10 wk (Fig. 4 C). In contrast, triple transgenic mice became hypoglycemic on average at 12.4 wk of age, a statistically significant delay compared with P14/RIP-Tag2 mice ($P < 0.005$). This spontaneous antitumor activity in triple transgenic mice indicates that cross-presentation of TAAs can generate limited effector function which can be detected in the presence of a high frequency of tumor-specific T cells.

Tumor-specific T Cells Do Not Show Evidence of Tolerance Induction. It has been observed that cross-presentation of self-antigens can lead to tolerance of antigen-specific T cells (24, 25). Studies have also shown that T cell tolerance is preceded by expansion and activation of effector function in many models (45–47). Thus, we wanted to test whether the increase in tumor burden lead to the induction of T cell tolerance in our model.

To assess whether tumor-specific T cells were tolerized by clonal deletion, PDLNs were isolated from tumor-bearing P14/RIP(GP × Tag2) triple transgenic mice and con-

trol mice, and the proportion of tumor-specific T cells determined by flow cytometric analysis (Fig. 5 A). We were unable to detect a reduction of tumor-specific T cells, as the percentage of P14 transgenic T cells in PDLNs of triple transgenic mice was similar to P14, P14/RIP-GP, and P14/RIP-Tag2 mice. Analyses of NDNLs and spleens also revealed normal proportions of tumor-specific T cells.

Another mechanism of T cell tolerance is the induction of unresponsiveness (48). T cells from the PDLNs of P14/RIP(GP × Tag2) triple transgenic mice were assayed for their proliferative responses to tumor-specific peptide in vitro. Proliferative responses of PDLN T cells to GP33 peptide from 6 of 7 triple transgenic mice were similar to controls (Fig. 5 B). In addition, GP33-induced proliferation of T cells from NDNLs and spleens of triple transgenic mice was normal (Fig. 5 C and data not shown, respectively). In 1 of 7 triple transgenic mice, the proliferative response of PDLN T cells was reduced to $\sim 30\%$ of the normal level, and was partially rescued by addition of conA supernatant (data not shown). However, T cells from other peripheral LNs of this particular mouse showed normal GP33-induced proliferation (data not shown). In general, the proliferation data indicate that tumor-specific T cells remain responsive both systemically and locally in the regional LN.

Although these results are consistent with a lack of T cell deletion and anergy, it remains possible that a subset of tumor-specific T cells was in fact deleted or anergized, and the production of a large number of tumor-specific T cells by the thymus in TCR transgenic mice replenished the deleted repertoire or compensated for any unresponsive P14 T cells. To address this possibility, we examined the fate of adoptively transferred P14 T cells in tumor-bearing, nonP14 transgenic hosts over time. Vα2⁺ CD8⁺ T cells in

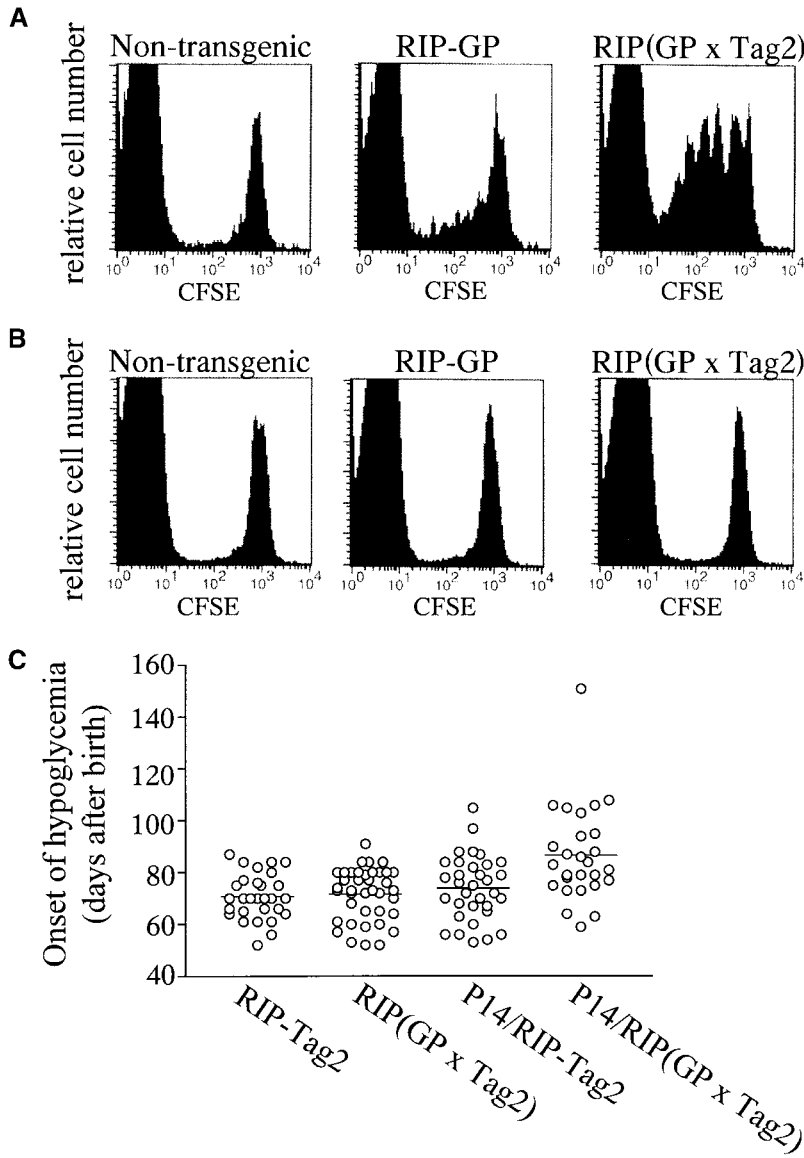


Figure 4. Spontaneous induction of proliferation and effector function of tumor-specific T cells. (A and B) CFSE-labeled, naive P14 transgenic T cells were adoptively transferred into nontransgenic, RIP-GP, or tumor-bearing RIP(GP × Tag2) hosts. 72 h later, lymphocytes from PDLNs (A) and NDLNs (B) were gated on Vα2⁺ lymphocytes and CFSE profiles determined. Profiles are representative of 5–7 mice from each genotype, from three independent experiments. (C) The age at which blood glucose levels dropped to or below 5 mM was recorded for RIP-Tag2, RIP(GP × Tag2), P14/RIP-Tag2, and P14/RIP(GP × Tag2) mice. Individual mouse data are shown, the horizontal bar indicates the mean onset of hypoglycemia.

PDLNs and NDLNs were enumerated at 1, 2.5, and 6 wk after transfer of 5×10^6 P14 T cells into RIP(GP × Tag2) and control hosts (nontransgenic, RIP-GP, and RIP-Tag2). Interestingly, the number of P14 T cells recovered from the PDLNs of RIP(GP × Tag2) hosts was increased compared with control hosts at all time points (Fig. 6 A). The highest number of P14 T cells was observed in the PDLNs after 1 wk and was maintained at a fairly constant cell number at 2.5 and 6 wk. This tumor-dependent expansion of tumor-specific T cells was specific to the PDLNs, as the number of P14 T cells recovered from NDLNs was similar in all genotypes at all time points (data not shown). Thus, in this situation where there were a restricted number of tumor-specific T cells, we did not detect deletion and in fact observed enhanced numbers of these T cells in PDLNs compared with antigen-negative and/or tumor-free mice at 6 wk after adoptive transfer. In addition, a comparison between pancreatic sections from 1

and 6 wk time points revealed an increase in CD8⁺ T cell islet infiltration in RIP(GP × Tag2) hosts over time (Fig. 6 B). Thus, the approximate twofold decline of total Vα2⁺ CD8⁺ T cells in the PDLNs from 1 to 6 wk correlated with an approximate twofold increase in cells in the pancreatic islets. Together these data demonstrate that expansion of P14 T cells was seen only in the presence of the tumor and only in the PDLNs. There was no evidence for deletion of tumor-specific T cells, but rather, a clear migration to the tumor in the pancreas.

Using the adoptive transfer system, we also assessed whether tumor-specific T cells remained functional at 3 and 6 wk time points. We have previously identified a novel peptide, MB6, which is recognized only by activated P14 TCR transgenic T cells, and not CTLs derived from LCMV-infected C57Bl/6 mice (49). After adoptive transfer of P14 T cells, hosts were challenged with LCMV, and 8 d later, PDLN cells, NDLN cells, and sple-

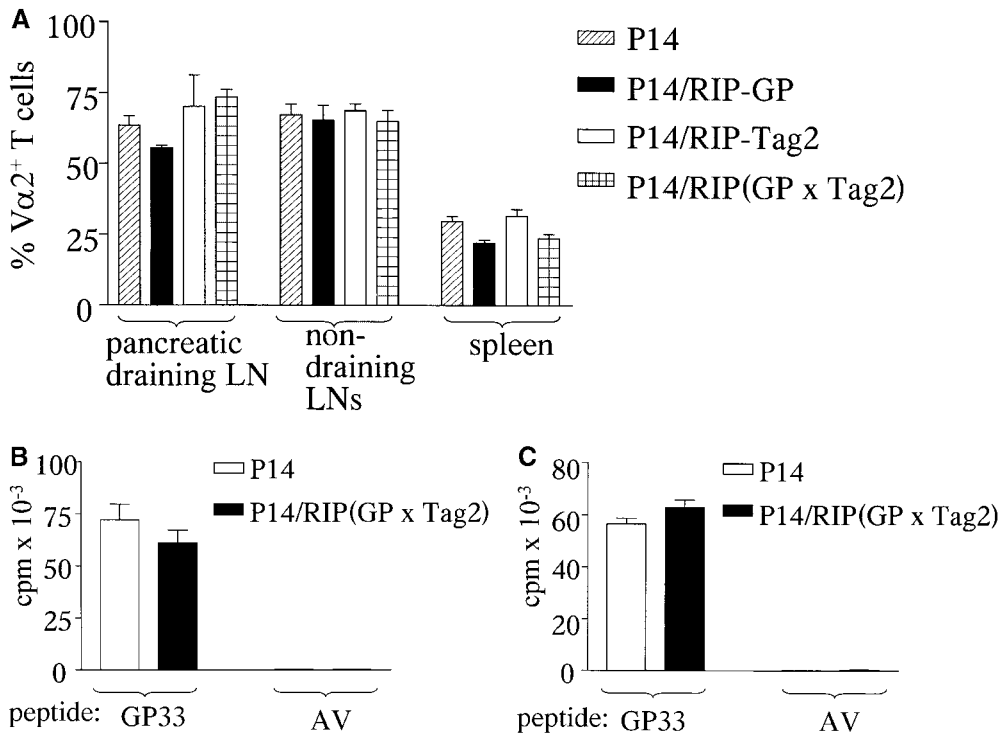


Figure 5. No significant tolerance induction of tumor-specific T cells in P14/RIP(GP × Tag2) mice. (A) Deletion was assessed by isolating PDLNs, NDLNs, and spleens from P14, P14/RIP-GP, tumor-bearing P14/RIP-Tag2, and P14/RIP(GP × Tag2) mice. The percentage of P14 transgenic was determined by the percentage of Vα2⁺ of lymphocytes by flow cytometric analysis. Data shown are the mean and standard deviation of 3–7 mice per genotype. (B and C) Anergy was assessed by proliferation assay of PDLN cells (B) and NDLN cells (C). Lymph node cells were incubated with C57Bl/6 spleen cell stimulators prepulsed with 10⁻⁷ M GP33 or negative control peptide AV. Data shown are from two mice per genotype, and results are representative of four experiments.

nocytes were assayed for CTL activity against the wild-type GP33 peptide and the altered peptide ligand, MB6. Thus, the generation of lytic activity against MB6 indicates a continued presence and functionality of adoptively transferred P14 T cells. Data in Table II show that, as expected, T cells from nontransgenic (C57Bl/6) mice without adoptive transfer did not lyse MB6-coated targets after LCMV immunization. In contrast, both local and systemic T cells from RIP(GP × Tag2) hosts, immunized with

LCMV 6 wk after adoptive transfer of P14 T cells, were able to lyse MB6-coated targets. This demonstrates that functional P14 tumor-specific T cells remained in the tumor-bearing mice. The lack of expansion of tumor-specific T cells we observed in PDLNs of nontransgenic hosts relative to RIP(GP × Tag2) hosts (Fig. 6 A) is consistent with the finding that T cells from PDLNs of nontransgenic hosts immunized with LCMV 6 wk after adoptive transfer did not lyse MB6-coated targets (Table II). T cells

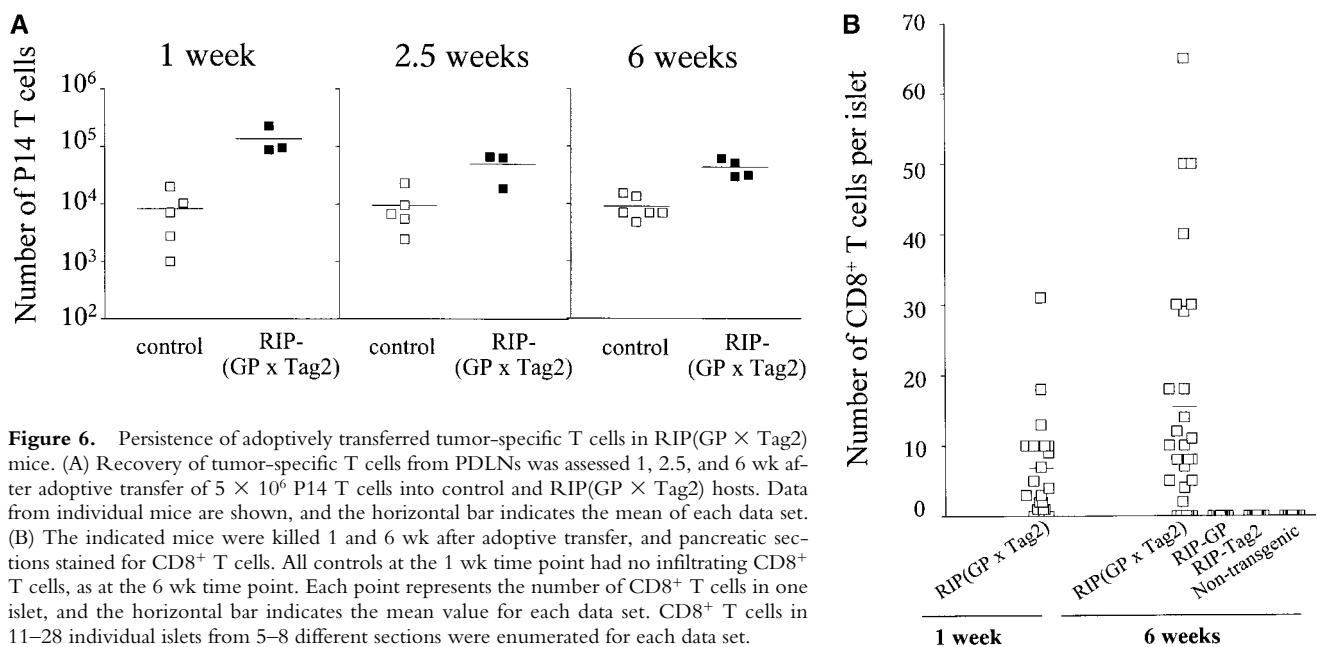


Figure 6. Persistence of adoptively transferred tumor-specific T cells in RIP(GP × Tag2) mice. (A) Recovery of tumor-specific T cells from PDLNs was assessed 1, 2.5, and 6 wk after adoptive transfer of 5 × 10⁶ P14 T cells into control and RIP(GP × Tag2) hosts. Data from individual mice are shown, and the horizontal bar indicates the mean of each data set. (B) The indicated mice were killed 1 and 6 wk after adoptive transfer, and pancreatic sections stained for CD8⁺ T cells. All controls at the 1 wk time point had no infiltrating CD8⁺ T cells, as at the 6 wk time point. Each point represents the number of CD8⁺ T cells in one islet, and the horizontal bar indicates the mean value for each data set. CD8⁺ T cells in 11–28 individual islets from 5–8 different sections were enumerated for each data set.

from all mice at each time point efficiently lysed GP33-coated targets (data not shown). Similar results as described above were observed at the 3 wk time point (data not shown).

Thus, no deletion or anergy of high avidity tumor-specific T cells occurred in P14/RIP(GP × Tag2) triple transgenic mice, or upon adoptive transfer into RIP(GP × Tag2) mice. In fact, local expansion of functional tumor-specific T cells was observed upon adoptive transfer into tumor-bearing transgenic hosts.

Administration of Tumor-specific Peptide and Anti-CD40 mAb Induces CTL Infiltration of Tumors, Tumor Regression, and Prolonged Animal Lifespan. To confirm that tumor-specific T cells in P14/RIP(GP × Tag2) triple transgenic mice could function in vivo, we attempted to elicit an antitumor response in vivo. We have previously reported that intravenous administration of GP33 peptide and agonistic anti-CD40 mAb into P14/RIP-GP mice results in the activation of P14 T cells, insulinitis, CTL-mediated pancreatic β cell destruction, and diabetes (50). Treatment of P14/RIP(GP × Tag2) triple transgenic mice with this same regimen resulted in infiltration of CD8⁺ T cells into insulinomas 5 d after peptide injection (Fig. 7 B). Infiltration was antigen-specific, as insulinomas not expressing LCMV-GP did not become infiltrated upon immunization (Fig. 7 A). MHC class I expression in insulinomas of triple transgenic mice was also markedly upregulated after peptide and anti-CD40 mAb treatment (Fig. 7 C and D). In addition to CTL infiltration, potent cytolytic effector function was generated: peptide and anti-CD40 mAb treated triple transgenic mice were rescued from hypoglycemia, reflecting destruction of tumorigenic β cells (Fig. 7 E). This antitumor activity resulted in prolonged animal lifespan, with triple transgenic animals surviving on average 10 wk longer after immunization compared with control mice not expressing LCMV-GP ($P < 0.0005$, Fig. 7 F). These results show that any undetectable T cell deletion that may have occurred in P14/RIP(GP × Tag2) mice was not sufficient to prevent priming of an antitumor response.

Antitumor Activity Is Elicited in NonTCR Transgenic Mice by Viral Immunization and Reimmunization. We have previously shown that a potent antitumor response is elicited in RIP(GP × Tag2) double transgenic mice upon immunization with LCMV (32). Together with the data presented in this study, this indicates that T cells are not significantly tolerized even in nonTCR transgenic tumor-bearing mice, with a normal endogenous repertoire of tumor-specific T cells. To further evaluate the responsiveness of endogenous tumor-specific T cells, we attempted to elicit antitumor activity by immunizing RIP(GP × Tag2) mice with a recombinant vaccinia virus expressing the LCMV-GP (vacc-G2) (37), which is a weaker CTL stimulus than LCMV (51). We found that animal survival was prolonged by a single immunization with vacc-G2, compared with immunization of RIP-Tag2 mice not expressing the LCMV-GP ($P = 0.006$) (Fig. 8). The ability of this weaker vacc-G2 stimulus to prime tumor-specific T cells further demonstrates that significant tumor-specific T cell tolerance did not occur in nonTCR transgenic mice.

Although immunization of RIP(GP × Tag2) mice with LCMV resulted in tumor regression, the antitumor response was transient, as the tumor itself was not sufficiently immunogenic to maintain tumor-specific CTL activity (32). To evaluate whether tumor-specific T cells were still functional in vivo after tumor relapse and consequently prolonged exposure to TAAs, we attempted to boost RIP(GP × Tag2) mice with vacc-G2 upon relapse of hypoglycemia. RIP(GP × Tag2) mice immunized with LCMV followed by a vacc-G2 boost (given on average 23 ± 2 d after primary LCMV immunization) had a statistically significant prolonged lifespan, compared with the same mice immunized with LCMV only (Fig. 8, $P < 0.005$). All RIP(GP × Tag2) mice succumbed to tumor relapse after secondary immunization with vacc-G2, suggesting that further immunizations would be required in order to maintain antitumor activity. The ability of repetitive immunization to further enhance animal survival indicates that in addition to a lack of significant T cell tolerance in

Table II. *Adoptively Transferred Tumor-specific T Cells Retain Functionality in RIP(GP × Tag2) Hosts*

Recipient genotype	Adoptive transfer of P14 T cells	PDLN cells		Splenocytes	
		MB6 targets	AV targets	MB6 targets	AV targets
Nontransgenic	–	5	5	2	7
Nontransgenic	+	5	2	23	0
RIP (GP × Tag2)	+	20	2	45	2
RIP (GP × Tag2)	+	18	2	35	2

5×10^6 P14 TCR transgenic T cells were adoptively transferred into the indicated recipient mice. 6 wk after transfer, all mice were infected with 2,000 PFU LCMV intravenously. 8 d after infection, PDLN cells and splenocytes were assayed for their cytotoxic activity against EL-4 targets pulsed with MB6 or negative control AV peptide. Data is expressed as the percentage of specific lysis at effector/target ratios of 60:1, with 10^4 effectors from spleens plated per well and only 5,000 effectors from draining LNs plated per well, due to the limited number of cells available in draining LNs. Spontaneous ^{51}Cr release was $<15\%$. Results are representative of data from two independent experiments.

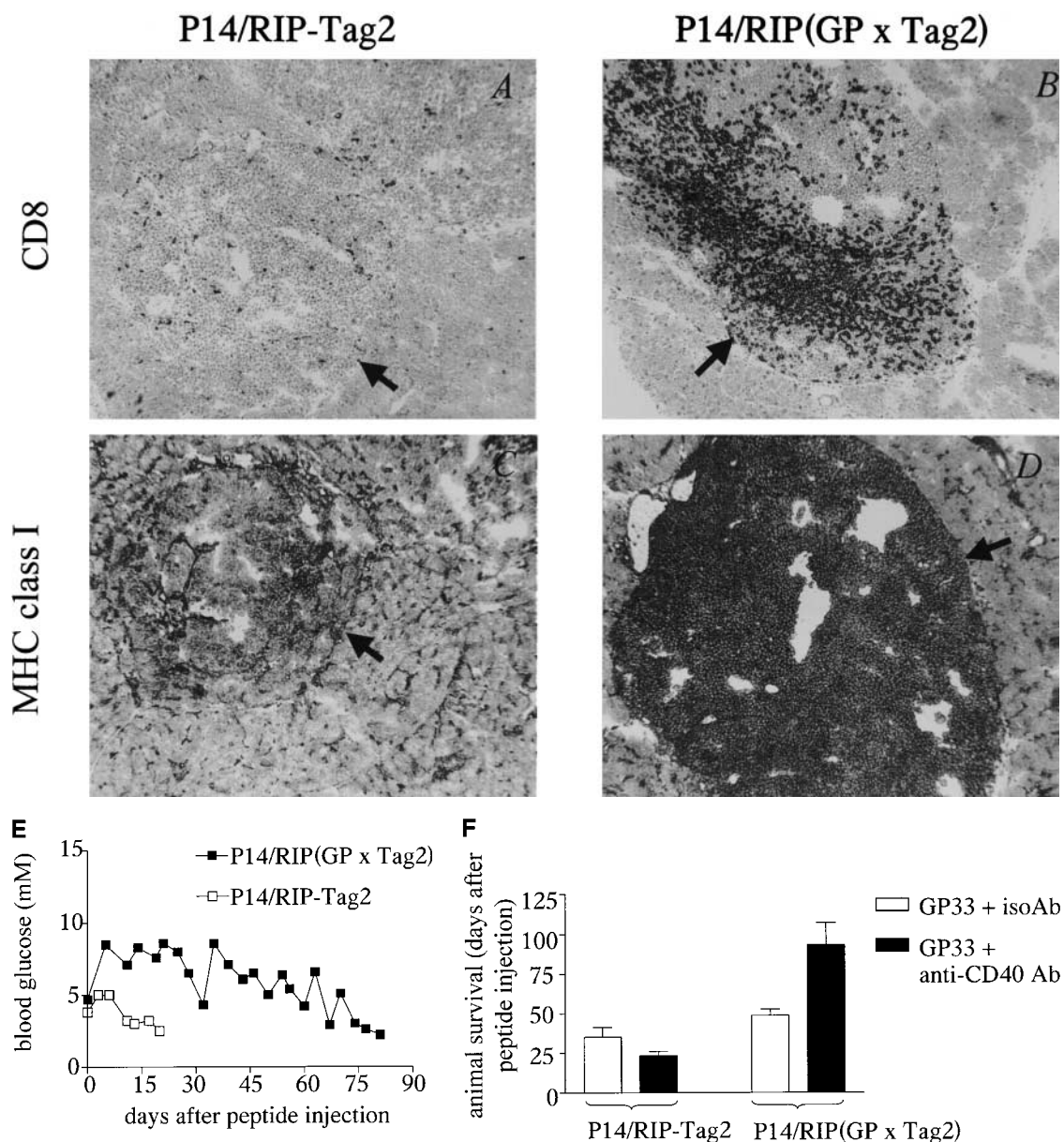


Figure 7. Active immunotherapy induces efficient antitumor CTLs in P14/RIP(GP × Tag2) mice. (A–F) Administration of tumor-specific peptide and anti-CD40 mAb induces antitumor activity. (A–D) Tumor-bearing P14/RIP(GP × Tag2) and P14/RIP-Tag2 mice were treated with 5 μg of GP33 peptide intravenously followed 2 d later with 100 μg of anti-CD40 mAb intravenously. Pancreata were harvested 5 d after peptide injection and sections were stained for CD8 (A and B) and MHC class I (C and D). Arrows indicate the location of tumors. Original magnification: 100×. (E) Representative blood glucose levels after peptide injection are shown. (F) P14/RIP(GP × Tag2) and P14/RIP-Tag2 mice were treated with 5 μg of GP33 peptide intravenously followed 2 d later with 100 μg of anti-CD40 mAb intravenously or 100 μg of isotype-matched control Ab intravenously. Mean and standard error of survival after peptide injection are shown of 3–7 mice per group.

naive tumor-bearing mice, tumor-specific T cells remained responsive despite an extended exposure to TAAs in vivo.

Discussion

Tumor Growth Enhances Activation of Tumor-specific T Cells in the Local Draining LNs. This study demonstrates that tumor growth leads to increased detection of TAAs by tumor-specific T cells. The impact of an increased tumor load is evident in the upregulation of CD44 and the induc-

tion of proliferation of CFSE-labeled tumor-specific T cells. In P14/RIP-GP mice, ~25% of P14 T cells upregulated CD44 expression (Fig. 2), and adoptive transfer of P14 T cells into RIP-GP mice resulted in limited proliferation (Fig. 4 A). In contrast, in P14/RIP(GP × Tag2) mice with an advanced tumor burden, an increased percentage of P14 T cells upregulated CD44 (~50%), and adoptive transfer of P14 T cells into RIP(GP × Tag2) mice resulted in vigorous proliferation. Thus, the effect of cross-presentation was enhanced with tumor growth.

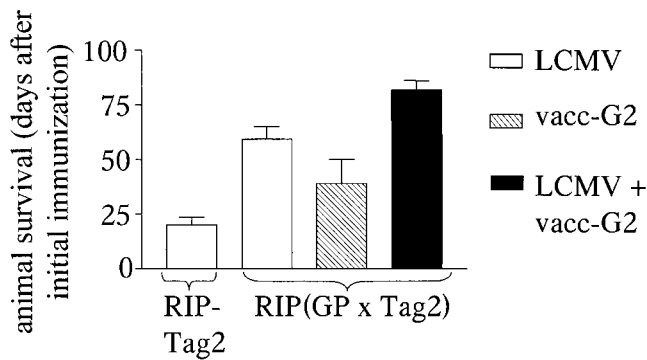


Figure 8. Viral immunization and reimmunization of RIP(GP × Tag2) mice elicits antitumor activity. RIP-Tag2 and RIP(GP × Tag2) tumor-bearing mice were immunized with 2,000 PFU LCMV intravenously, or 10⁶ PFU vacc-G2 intravenously, or were boosted with vacc-G2 upon tumor relapse after primary LCMV immunization. Mean and standard error of survival after initial immunization are shown of 5–14 mice per group.

T Cell Tolerance, Activation, and Immunosurveillance. Numerous studies have investigated whether the presence of a tumor leads to tolerance of tumor-specific T cells. Tumor-induced T cell tolerance has been reported to occur by different mechanisms in various situations, in both mouse models (4, 7, 11, 15, 52) and human studies (5, 8–10, 13). However, studies have also shown that tumor-specific T cells may become activated in the presence of a tumor, or they may remain ignorant of TAAs and consequently have the potential to be activated by immunotherapy (15, 53–59). We have addressed the issue of tumor-induced T cell tolerance in a model of endogenous tumors and defined TAAs. We have demonstrated that tumor-specific T cells encounter cognate antigen in tumor-bearing hosts, but did not find evidence for tolerance induction. In tumor-bearing P14/RIP(GP × Tag2) animals, the percentage of endogenous transgenic tumor-specific T cells and their proliferative capacity remains similar to tumor-free animals (Fig. 5). Cross-presented tumor antigens were able to promote the expansion of adoptively transferred high avidity tumor-specific T cells into tumor-bearing mice (Fig. 6 A and B). There was no evidence for tolerance since these T cells were present and remained functional to subsequent virus challenge (Table II). Previous studies have shown that virus immunization was unable to break LCMV-GP-induced tolerance of nondeleted T cells (46, 60). Therefore, the detection of P14-specific cytolytic T cells after LCMV challenge reflects the presence of functional P14 transgenic T cells, rather than the activation of tolerized cells or “breaking” tolerance. Thus, evidence from our studies demonstrate that high avidity tumor-specific T cells are not tolerized in vivo.

Many factors may influence the induction of T cell immunity versus tolerance. Generally, T cell tolerance is thought to occur when T cells encounter antigen on “immature” or resting APCs, while effective T cell activation is promoted through antigen-specific interactions with activated, mature APCs (61–66). An important component of

APC maturation is the generation of proinflammatory signals. Our data support a scenario where the high degree of cell death inherent in rapidly proliferating tumors, together with an increase in total mass, confers a proinflammatory environment, sufficient to subvert the induction of tolerance and promote inflammation and immunity. It is likely that many other factors contribute to the proinflammatory environment associated with the tumor, such as other cytokines including TNF- α .

Why then does T cell tolerance occur in some models? Although cross-presentation of tissue-specific antigens has been shown to lead to tolerance, these models expressed a defined self-antigen in tissues where inflammatory signals are not normally present (24, 25). Many studies that have observed T cell tolerance to tumors involve haematological or metastasizing tumors, intravenously administered tumor cells or examine tumor infiltrating lymphocytes (5, 6, 8, 9, 11, 13, 15, 67). In these cases, direct presentation may occur and induce tolerance, or perhaps the tumor is perceived as noninvasive and therefore does not promote inflammatory signals and “immunogenic” cross-presentation on activated APCs.

In contrast to models of transplanted tumors, the RIP-Tag2 model of spontaneously arising tumors of endogenous origin is well suited for examining issues concerning natural tumor immunosurveillance (68, 69). In our model, despite the overwhelmingly tumor-specific T cell repertoire in P14/RIP(GP × Tag2) animals and evidence for their activation by TAAs, immunosurveillance is inefficient in preventing tumor development. This is likely due to the limited degree of cross-presentation in the regional LNs, which would result in insufficient numbers of activated tumor-specific T cells to protect the host from tumor growth.

Induction of Antitumor Immunity. Recent developments in immunotherapeutic strategies include attempts to activate endogenous tumor-specific T cells by transfusion of mature dendritic cells presenting tumor-derived peptides (70–72). Maturation of dendritic cells results in their ability to efficiently activate naive CTLs, by virtue of the upregulation of a range of costimulatory, adhesion, and cytokine molecules (73). Among the dendritic cell maturation signals that have been identified is ligation of CD40 by its ligand, CD154 (74–77). We show here that intravenous treatment of P14/RIP(GP × Tag2) mice bearing advanced tumors with GP33 tumor-derived peptide and agonistic anti-CD40 mAb elicits a potent antitumor CTL response that results in greatly enhanced animal lifespan. This data, together with other studies that demonstrate antitumor immunity after in vivo ligation of CD40 (78–85), indicate that CD40-based therapeutic strategies present a practical alternative to strategies that rely on ex vivo generation of dendritic cells manipulated to express TAAs. The results presented here showed that all tumor-bearing mice immunized with GP33 and anti-CD40 mAb or viruses experienced tumor relapse. This indicates that therapeutic immunization with self-antigens must be done in a strong and repetitive manner in order to effectively control tumor growth.

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