Expression of a Tolerizing Tumor Antigen in Peripheral Tissue Does Not Preclude Recovery of High-Affinity CD8+ T Cells or CTL Immunotherapy of Tumors Expressing the Antigen

Claes Öhlén, Michael Kalos, Doley J. Hong, Aaron C. Shur and Philip D. Greenberg

*J Immunol* 2001; 166:2863-2870; doi: 10.4049/jimmunol.166.4.2863

http://www.jimmunol.org/content/166/4/2863

---

**References**  
This article cites 46 articles, 28 of which you can access for free at:  
http://www.jimmunol.org/content/166/4/2863.full#ref-list-1

**Subscription**  
Information about subscribing to *The Journal of Immunology* is online at:  
http://jimmunol.org/subscription

**Permissions**  
Submit copyright permission requests at:  
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**  
Receive free email-alerts when new articles cite this article. Sign up at:  
http://jimmunol.org/alerts
Expression of a Tolerating Tumor Antigen in Peripheral Tissue Does Not Preclude Recovery of High-Affinity CD8+ T Cells or CTL Immunotherapy of Tumors Expressing the Antigen 1

Claes Öhlén, Michael Kalos, Doley J. Hong, Aaron C. Shur, and Philip D. Greenberg

Transgenic (TG) mice were generated selectively expressing the gag protein of Friend murine leukemia virus (FMuLV) in the liver. FMuLV gag is also expressed by the FBL leukemia, and is the immunodominant tumor Ag of the CD8+ T cell response in C57BL/6 mice. gag-TG mice expressing FMuLV gag in the liver were tolerant to the protein and failed to generate a CTL response to either FBL or FMuLV gag. This tolerance reflected anergy rather than deletion, as CTL responsiveness could be recovered after four cycles of in vitro stimulation. Adoptively transferred gag-specific T cells were not anergized in gag-TG recipients, as revealed by antitumor activity in vivo. Also, such T cells did not induce detectable autoimmune injury in gag-TG liver cells. These results suggest that the requirements for a tissue Ag to provide a tolerizing stimulus are distinct from those for being the target of a T cell-mediated autoimmune response and that the requirements for induction and maintenance of peripheral tolerance are distinct for naive and primed T cells. That anergic T cells reactive with tumor-associated Ags can be recovered by repetitive in vitro stimulation and can mediate tumor therapy suggests strategies that use such Ags to generate CTL for adoptive immunotherapy should be further developed. The Journal of Immunology, 2001, 166: 2863–2870.

A major obstacle to the development of T cell therapy for human cancers has been the identification of suitable tumor Ags that can be recognized by peripheral T cells from the tumor-bearing host. The ideal tumor Ags are immunogenic proteins uniquely expressed by neoplastic cells. Despite evidence for such Ags in many experimental animal tumors as well as in occasional human cancers, most of the characterized human tumor Ags are more appropriately designated as tumor-associated, based on detectable expression in some normal cells as well as in tumor cells (1, 2). Enthusiasm for prospectively evaluating tumor proteins not restricted in expression to the tumor cell as targets for therapeutic antitumor T cell responses has been dampened largely by two perceived obstacles: 1) the host is likely to be tolerant to most tumor-associated Ags (TAA) because they are also normal self-proteins; and 2) if methodologies could be developed to break tolerance and elicit host T cell responses to the TAA, such responses might be pathogenic, mediating not only antitumor activity but also autoimmune damage to the normal tissues expressing the Ag. Despite these concerns, results from recent clinical vaccine trials that use peptide epitopes derived from TAA expressed in melanoma suggest it may be possible to achieve clinical benefit with little or no toxicity to patients (3–5).

Tolerance to self-proteins has been evaluated in several distinct murine models. Intrathymic expression of a self-protein usually leads to deletion of potentially self-reactive T cells (6–8), whereas expression of the Ag in peripheral (extrathymic) tissues often induces tolerance by nondeletional mechanisms, leading to clonal nonresponsiveness or anergy (9–13). In some settings with peripheral expression of the Ag, the function of anergic self-reactive T cells has been recoverable, suggesting that these cells could potentially be available for use in tumor therapy (12–15). An alternative nondeletional outcome for CD8+ T cells that are specific for a peripherally expressed Ag has been defined as ignorance (16). Mice expressing an lymphocytic choriomeningitis virus (LCMV)-gp transgene in the pancreas showed no signs of autoimmunity and possessed the same T cell response to LCMV-gp after stimulation as wild-type mice. However, the T cells triggered by LCMV infection were no longer ignorant of the normal peripheral tissue and mediated rapid destruction of LCMV-gp-positive pancreatic islet cells leading to diabetes. These data suggest that a low level of a self-Ag in a noninflammatory milieu can be ignored by naive CTL, but raise the concern that activation of the T cells, such as by intentional immunization to a TAA, may alter the threshold for triggering and result in recognition of previously ignored normal tissues and autoimmune injury.

Studies in humans have demonstrated that T cells that recognize epitopes from normal proteins expressed in tumors, including both lineage-specific proteins and overexpressed cellular oncopogens, can be isolated in vitro (1, 17–19). However, this does not preclude in vivo anergy induction and deletion, as the recovered T cells may be of too low affinity to recognize either tumor or normal targets (20). Thus, models are necessary to define the nature of responsiveness to peripherally expressed TAA, determine the conditions by which nonresponsiveness can be overcome, and elucidate which categories of TAA can be safely and effectively used as targets for T cell therapy of tumors.
We describe here a C57BL/6 (B6)-transgenic (TG) mouse model in which the gag protein from the oncogenic Friend murine leukemia virus (MuLV) is expressed under the control of the mouse albumin promoter in the liver. This MuLV gag protein is also expressed by FBL, an MuLV-induced erythroleukemia of B6 origin, and is the immunodominant target of the CD8+ T cell response in B6 mice to the tumor (21, 22). CD8+ T cells recognizing MuLV gag can mediate complete eradication of disseminated leukemia following adoptive transfer into tumor-bearing B6 mice, as long as CD4+ T cell help or exogenous IL-2 is provided to sustain the CD8+ response (23, 24). By using the gag-TG mice, we describe a model for immunotherapy against a tolerizing self-tumor Ag and characterize the consequences of transferring tumor-reactive CD8+ T cells that also recognize the transgene as treatment of disseminated leukemia.

Materials and Methods

Generation of MuLV gag TG mice and analysis of MuLV gag tissue expression

The construct CB16 containing the MuLV gag sequence from MuLV clone 57 has been described previously (25). In this construct, a CGT start site at nt 325 encoding glycosylated MuLV gag and the ATG start site at nt 619 encoding gag have been mutated to prevent translation initiation, and the ATG site at nt 595 encoding gag was mutated to prevent translation initiation from this site (25, 26). This construct was selected because the immunodominant CD8+ response to MuLV gag in B6 mice is contained in the gag protein (22). An expression construct, pAlb-int-gag359, was generated in which the MuLV gag sequence from CB16 was placed under the control of the albumin enhancer/promoter and the rat insulin intron A fragment inserted to improve expression (27). A construct, pAlb-int-gag590, was generated by the following sequential molecular manipulations: excision of a 2279-bp SspI/Stul fragment from the CB16 construct, blunt-ending, and subcloning the fragment into the Alb-hg vector (28) previously digested with BamHI and SmaI (to excise the high gc gene). Correct orientation of the gag sequence relative to the albumin promoter enhancer was determined by restriction analysis. To generate Alb-int-gag590, a 150-bp rat insulin intron A fragment was excised from the plasmid pUC18/rat insulin II intron A using BamHI and BglII and ligated into the plasmid pAlb-int-gag590 that had been digested with BamHI. Correct orientation of the insert was determined by restriction analysis.

For injection into mice, pAlb-int-gag was digested with KpnI and ClaI to release the expression cassette from the vector backbone, electrophoresed onto a 1.2% agarose gel, and the sequence purified using QIAEX II (Qiagen, Chatsworth, CA). The DNA was resuspended in endotoxin-free deionized H2O, quantified, and injected into mouse embryos by using standard techniques. MuLV gag-positive founder mice, as well as subsequent gag-positive offspring, were identified by analysis of tail DNA by using standard PCR protocols with MuLV gag-specific primers (forward, 5'-GACTAAGCTGTGACCGGGGA-3'; reverse, 5'-GATCTCTACCGAGGTGGTGTGG-3'). Expression of MuLV gag in gag-TG and control B6 mice was analyzed by semiquantitative RT-PCR of RNA from the kidney, liver, spleen, and thymus of adult mice. Tissue fragments were snap-frozen on removal from mice, and mRNA was isolated and purified from the tissue fragments as well as positive controls (4 × 106 tissue in vitro FBL cells) and negative (water) controls, with the Strataprep Total RNA Miniprep Kit (Stratagene, La Jolla, CA). The matrix-bound, isolated mRNA was digested with DNase I to eliminate potential contaminating DNA. Reverse transcription of the purified mRNA was performed with the Thermoscript RT-PCR System (Life Technologies, Grand Islan, NY). First-strand synthesis of isolated mRNA was achieved with Plasmid Tag Polymerase (provided in the Thermoscript RT-PCR System) and custom actin primers (forward, 5'-GTGGGAGCCCCAGGCCACCA-3'; reverse, 5'-GATCTCTACCGAGGTGGTGTGG-3'). First-strand products were normalized by titration of starting material based on the band intensity of the amplified β-actin. PCR amplification of MuLV gag was conducted on the normalized first-strand products by using the custom MuLV gag primers. PCR products were then run out on a 2% agarose gel. The PCR protocols for the amplification of β-actin and MuLV gag were identical: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for each of 34 cycles.

The presence of MuLV gag protein in the liver of gag-TG mice was assessed from snap-frozen liver tissue that was lysed in lysis buffer, run on a 12% SDS-PAGE gel, and blotted to nitrocellulose filter. After blocking in 5% milk, the filters were probed with supernatant from the rat hybridoma R187 (a kind gift of B. Chesbro, Rocky Mountain Laboratories, Hamilton, MT (29)), washed, and specific binding detected by incubation with secondary HRP-conjugated anti-rat Ab and ECL Western blotting detection reagents (Amer sham Pharmacia, Piscataway, NJ).

Cell Lines, Medium, and Peptides

FBL-3, a Friend virus–induced erythroleukemia of B6 (H-2b) origin, expresses MuLV env- and gag-encoded products and MHC class I molecules but does not express MHC class II molecules (23). E10 is a MuLV negative subline of the B6 EL-4 thymoma. The mapping and the sequence (CCLC/LTVFL) of the dominant H-2Dd restricted MuLV gag epitope in FBL in B6 mice has been described (22), and the peptide was synthesized at the University of Washington (Seattle, WA). Unless otherwise stated, all cell culture was performed in RPMI 1640 supplemented with antibiotics, 2-ME, and 10% FCS.

Maintenance and immunization of mice

B6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The transgenic B6 mice were established and bred in our animal facility, and all mice were maintained under specific pathogen-free conditions. Six- to 8-wk-old mice were immunized twice with 5 × 106 irradiated FBL (10,000 rad) cells intraperitoneally (i.p.) 2 wk apart. Immune spleenocytes were harvested 2–3 wk after the second immunization.

Adoptive chemoimmunotherapy (ACIT)

Adoptive immunotherapy experiments were performed as previously described (23). Briefly, on day 0, recipient mice were inoculated i.p. with 5 × 106 FBL leukemia cells. On day 5, after the tumor cells have widely disseminated, mice were treated with 180 mg/kg cyclophosphamide (CYP) i.p., followed in 6 h by adoptive transfer of splenic donor cells from immune mice, or of FBL-reactive CTL lines or clones grown and expanded in vitro, administered i.p. Spleenocytes administered as effector cells were first depleted of CD4+ cells using Dynabeads (Dynal, Lake Success, NY) according to the manufacturer's protocol, and the contaminating CD4 cells were always < 2% of the injected T cell population. T cells grown in vitro were analyzed by FACS and confirmed to be essentially 100% CD8+.

Analysis of autoimmune injury

Groups of gag-TG and B6 mice treated with ACIT as above were sacrificed at 2–14 days after T cell transfer. Liver samples were fixed in formaldehyde, paraffin-embedded, sectioned, and stained with hematoxylin/eosin. Coded specimens were analyzed for inflammation and lymphocyte infiltration by a reference pathologist (Phoenix Central Laboratories, Everett, WA). Concurrent peripheral blood samples were analyzed for alanine aminotransferase and aspartate aminotransferase as biochemical indicators of liver injury (Phoenix Central Laboratories).

Generation and testing of FBL-specific T cells

A total of 2.5 × 106 immune spleenocytes from B6 or gag-TG mice were stimulated with 5 × 106 irradiated FBL cells for 5 days in T25 tissue culture flasks. After 5 days, the CTL were tested in standard 3Cr release assays against labeled FBL. E10 cells alone, or E10 pulsed with MuLV gag peptide at the indicated concentrations. CTL lines were generated in vitro by repetitive stimulation of 2 × 106 cultured cells with 2 × 106 irradiated (10,000 rad) FBL in T25 flasks containing 5 × 106 irradiated (2,000 rad) B6 spleenocytes as feeder and 20 μM IL-2 in a total volume of 10 ml. Lines were tested for cytolytic activity 5 days after an in vitro stimulation.

Results

Generation of MuLV gag TG mice

The single MuLV gag open reading frame codes for two alternative translational products: Pr65gag and gp75gag. Transcription of the larger gag protein gp75gag is initiated from a CTG start site at position 355 of the MuLV sequence, which is upstream and in-frame with the ATG start site for the conventional gag protein Pr65gag at nt 619. The higher molecular weight, which has been named...
glycogag, has an additional 98-aa N-terminal leader sequence preceding the amino acid sequence of Pr65<sup> gag</sup> (25, 26). Because translation is predominantly initiated at the ATG start site at nt 619, the major protein species generated from this open reading frame is Pr65<sup> gag</sup> (25). However, the dominant CTL epitope elicited in B6 mice in response to FMuLV<sub> gag </sub>is located in the N-terminal leader segment of the FMuLV gPr75<sup> gag</sup> protein, encoded by FMuLV nt 577–603 (22). Therefore, the gag transgene was designed to optimize transcription and expression of this immunodominant CTL epitope. An FMuLV<sub> gag </sub>gene was constructed (kindly provided by Dr. Jean-Luc Darlix, Lyon, France) in which the CTG start site for Pr65<sup> gag</sup> at nt 355 was mutated to an ATG, and the ATG start site for gPr75<sup> gag</sup> at nt 619 mutated to an AGC (Fig. 1A). In this construct, translation has been demonstrated to occur almost exclusively from nt 355 (25). The construct was subcloned into a vector optimized for selective expression in the liver of transgenic mice off an albumin promoter (kindly provided by Dr. Richard Palmiter, Seattle, WA) (28) (Fig. 1B). The construct was microinjected into pronuclei of fertilized eggs from B6 mice according to standard protocols. A founder positive for the transgene was identified by PCR of tail DNA (data not shown), which transmitted the transgene to subsequent generations.

FMuLV<sub> gag </sub>expression in mice was analyzed from cDNA generated from mRNA isolated from kidney, liver, spleen, and the thymus of gag-TG and normal B6 mice (Fig. 2 top panel), with the amount of cDNA normalized to β-actin as control (Fig. 2 lower panel). A robust band of the expected size (417 bp) was amplified from cDNA generated from liver tissue of gag-TG mice, whereas cDNA from the liver of nontransgenic littermates was negative. Considerably weaker bands also were detected in other tissues from gag-TG after 34 cycles of PCR, suggesting some leakiness of the promoter. These bands did not appear to result from potentially contaminating DNA, as amplification of RNA samples mock treated without reverse transcriptase failed to generate a signal (data not shown). No FMuLV<sub> gag </sub>mRNA expression was detected in tissues from B6 mice.

Liver cells were next analyzed for expression of the gag protein by western blotting. Liver tissue from B6 and gag-TG mice was lysed in lysis buffer and resolved on SDS-PAGE. The lysates were transferred to a nitrocellulose filter, and probed with an FMuLV<sub> gag </sub>reactive mAb (Fig. 3). The lane containing the FBL tumor lysate had a strong band of 65 kDa size as well as a weak band at ~75 kDa, presumably reflecting both the Pr65<sup> gag </sup>and gPr75<sup> gag </sup>. Neither band was detectable in lysates from normal B6 mice. The gag-TG liver lysate had a single band at ~75 kDa, consistent with the modification of the transgenic construct to translate only the larger protein species.

Peripheral expression of FMuLV<sub> gag </sub>abolishes the anti-FBL CTL response in B6 mice

To investigate the immunological consequences of expressing FMuLV<sub> gag </sub>as a TAA, gag-TG and normal B6 mice were primed and boosted in vivo with irradiated FBL. Two weeks after the second immunization, spleens were harvested, stimulated in vitro with irradiated FBL, and tested for the presence of FBL-reactive CTL. After in vitro sensitization for 5 days, spleen cells from in vivo-primed normal B6 mice exhibited specific lytic activity for FBL (Fig. 4). This CD8<sup>+</sup> T cell mediated (Ref. (21) and data not shown) cytotoxic activity recognized the dominant CTL epitope, as demonstrated by pulsing FMuLV- negative targets with the peptide defining this gag epitope. In contrast, no CTL activity to FBL or to targets pulsed with the immunodominant epitope could be
detected in cultures derived from gag-TG mice. Thus, the gag-TG mice are not only tolerant to FMuLVgag but also fail to generate CTL responses to any potentially subdominant MHC class I-restricted epitopes encoded by other genes in FBL.

Recovery of fully functional CTL after repeated in vitro stimulation

In a clinical setting, adoptive therapy will require a means to recover and expand potentially tolerant T cells reactive with the TAA. The failure to detect a CTL response in gag-TG animals could be the result of anergy, from which recovery would potentially be possible, or from permanent deletion of the FMuLVgag-specific repertoire. Therefore, we attempted to overcome tolerance and expand FMuLV-reactive CD8+ T cells by removing the cells from the tolerogenic in vivo environment and repetitively stimulating the cells in vitro in the presence of supplemental IL-2.

Mice were primed and boosted in vivo with irradiated FBL, and their splenocytes then stimulated weekly in vitro with irradiated FBL and IL-2. Cell cultures from primed B6 mice expanded with each stimulation cycle, but the cultures from gag-TG mice exhibited an initial contraction, followed by stable cell numbers, and then proliferation after three cycles. Analysis of cytolytic activity after the fourth cycle revealed FBL-reactive CTL in T cell lines from gag-TG animals that killed FBL targets of cytolytic activity after the fourth cycle revealed FBL-reactive cell numbers, and then proliferation after three cycles. Analysis of in vitro cytotoxicity, CD4-depleted splenocytes from gag-TG mice were unable to eradicate the FBL tumor from B6 mice. However, gag-specific CD8+ CTL recovered from gag-TG animals by repetitive in vitro stimulations exhibited efficacy in adoptive therapy similar to the CTL lines established from normal B6 mice (Fig. 6).

Expression of FMuLVgag in the liver does not affect the efficacy of transferred FBL specific CTL, or serve as a target for autoimmune injury

Even if T cells reactive with a TAA can be rescued as above, such T cells might still not be useful for therapy. First, the mechanisms operative for maintaining tolerance in gag-TG mice could potentially similarly induce anergy in transferred T cells specific for the TAA and interfere with efficacy. Second, if the tolerizing signals lytic activity by CTL from gag-TG and normal B6 mice were observed over peptide titrations from 0.01 to 10 μg/ml (Fig. 5). Repeat experiments with additional CTL lines at ranging peptide doses suggested no reproducible differences between the CTL lines. Although other factors could contribute to the avidity of these CTL lines for the target, the peptide titration studies suggest that the CTL derived from gag-TG mice are of relatively similar affinity to those from B6 mice. These results suggest that the CTL recovered from the tolerant mice have a repertoire containing some high-affinity and not just residual low-affinity T cells. The recovery of such higher-affinity T cells after repetitive in vivo stimulation likely reflects the reversal of anergy in tolerant cells in this environment rather than just outgrowth of rare T cells that have not undergone deletion, as repetitive in vivo stimulations in the tolerant mice failed to result in detectable expansion of gag-reactive T cells (data not shown).

To further evaluate the activity of the recovered gag-specific cells, the cells were tested in adoptive immunotherapy experiments, in a model that requires in vivo proliferation and persistence of the transferred cells for tumor eradication (30). B6 mice were inoculated with FBL tumor cells i.p. on day 0, and on day 5 were treated with CY followed in 6 h by transfer of 107 FMuLVgag-specific CD8+ T cells. Low doses of IL-2 (104 U/day) were provided on days 5–19 to support proliferation and survival of the transferred CD8+ T cells. This specific ACIT regimen has been shown previously to cure the vast majority of mice bearing established disseminated FBL tumor (24). As suggested by analysis of in vitro cytotoxicity, CD4-depleted splenocytes from gag-TG were unable to eradicate the FBL tumor from B6 mice. However, gag-specific CD8+ CTL recovered from gag-TG animals by repetitive in vitro stimulations exhibited efficacy in adoptive therapy similar to the CTL lines established from normal B6 mice (Fig. 6).
prove incapable of energizing infused effector T cells, these CTL, which are targeted to a protein shared between the tumor and normal tissues, could potentially lead to severe autoimmunity. Therefore, B6 and gag-TG mice were inoculated with FBL tumor cells on day 0, and received treatment on day 5 with cyclophosphamide followed in 6 h by transfer of $10^7$ FMuLV-gag-specific CTL derived from a B6 mouse. The mice then received on days 5–19 $10^6$ U IL-2/day i.p. FBL tumor was eradicated by this treatment regimen in both the gag-TG and B6 mice (Fig. 7). Thus, the FMuLV gag expressed in the liver did not interfere with the in vivo antitumor activity or tolerize primed activated CTL, at least during the 4-wk time required for tumor eradication (30). The results suggest that primed T cells have a different threshold for tolerance induction than naive T cells.

The persistence and function for several weeks of transferred effector CTL could result in autoimmunity to liver cells expressing the FMuLV gag protein. Therefore, blood was drawn from mice at multiple time points after T cell therapy of gag-TG and B6 mice, and serum levels of the liver enzymes AST and ALT measured as indicators of liver injury. Additionally, cohorts of treated mice were sacrificed intermittently from days 7–26 for histological analysis of liver tissue. All B6 and gag-TG animals cured of disseminated tumor by adoptive transfer of FMuLV gag-specific CTL appeared healthy. Moreover, neither histologic analysis of liver sections (representative sections obtained on day 12, 1 wk after T cell therapy and IL-2 administration, are shown in Fig. 8) nor measurement of AST and ALT serum levels (data not shown) at any time point revealed evidence of autoimmune injury in treated gag-TG animals.

**Discussion**

FBL is a retrovirally transformed leukemia expressing high levels of an immunogenic viral protein, FMuLV gag, that induces an immunodominant CTL response that can eradicate the FBL tumor (22, 24). The transgenic mice described in this report express the gag gene in the liver under the control of the albumin promoter, rendering this potentially immunogenic tumor protein no longer tumor specific. These mice have provided the opportunity to examine the consequences on CTL function and on tumor therapy of the expression of an immunodominant tumor Ag in peripheral tissues. The results presented in this report demonstrate a remarkably selective activity of effector T cells for the tumor and not normal cells expressing the FMuLV gag protein.

A major obstacle to pursuing T cell therapy targeting tumor Ags that also are expressed in normal tissues is the difficulty in isolating such T cells, which is at least in part attributable to tolerance in the host. This obstacle was evident in the gag-TG mice, as initial efforts to immunize the mice with FMuLV gag, in vivo to expand the number of gag-specific CTL were unsuccessful. However, FMuLV gag-reactive T cells with efficacy in tumor therapy similar to nontolerant T cells from immunized B6 mice could be recovered from gag-TG mice after removal from the in vivo tolerizing environment. The approach employed, involving repeated cycles of in vitro stimulation in combination with supplemental IL-2, is analogous to methods we have used to isolate human melanoma-reactive T cells specific for normal melanocyte Ags from patients with melanoma (31).

Other studies that used nominal Ags have also suggested that maintenance of the anergic state in self-reactive T cells requires continuous exposure of the T cells to the tolerogen, and that the nonresponsive state can be reversed after withdrawal of the tolerogen. (15, 32, 33). However, the persisting recovered T cells may have a lower affinity than T cells isolated from hosts not expressing the Ag. In a transgenic model in which hemagglutinin from influenza virus was expressed in islet cells in the pancreas, the recovered T cells recognized a transfected renal carcinoma cell line despite exhibiting only low affinity for hemagglutinin (34). These CD8+ T cells were capable of rejecting the tumor in vivo without causing detectable autoimmune injury. Thus, nontolerant low-affinity T cells reactive with TAA may be isolated in settings in which self-peptides are expressed in the liver under the control of the albumin promoter.
B6 and gag-TG mice were inoculated on day 0 with $5 \times 10^6$ live FBL cells i.p. Groups of mice were either left untreated or treated 5 days later with 180 mg/kg CY, followed in 6 h by transfer of $10^7$ cloned FMuLVgag-specific CD8+ CTL, or with control B6 splenocytes. All treated mice received $10^6$ U of IL-2 i.p. beginning on day 5 immediately after cell transfer through day 19.

Another potential obstacle for therapy with tumor-reactive T cells that also recognize normal tissues is that the infused T cells might be tolerized after transfer and fail to mediate antitumor activity. However, the therapeutic efficacy observed after transfer of FMuLVgag-reactive primed T cells suggest that the requirement for tolerizing primed T cells is distinct from the requirements for tolerizing naive T cells. This appears to be the reciprocal of the requirements for activation of primed T cells, which requires lower levels of Ag and is less dependent on costimulatory and accessory molecules than activation of naive T cells (35, 36). Thus, a tolerizing signal for naive T cells may be perceived as a competent activating signal by primed T cells.

If the tolerizing peripheral tissue can actually activate primed but not naive T cells, then transfer of primed cells would be expected to mediate acute and/or chronic injury to the normal tissue expressing the proteins. However, histological and biochemical analysis of liver tissue from gag-TG mice that received immune T cells that were capable of rejecting a tumor challenge, an activity that requires retention of function for at least 30 days (30), failed to reveal evidence of liver injury. Thus, despite expression of FMuLVgag in the liver adequate to induce nonresponsiveness in naive T cells, activated effector cells did not promote injury to normal tissue. This may reflect differential susceptibilities of normal tissues and the tumor to the cytotoxic effector mechanisms and/or be a consequence of quantitatively different levels of Ag expression. Other murine models have also suggested that normal tissues may be more resistant to injury than tumor cells (34, 37, 38). We previously reported that transgenic mice expressing from an Ig promoter the FMuLV-env gene, which is the target for an MHC class II-restricted FBL-specific CD4+ T cell response, were resistant to autoimmune injury despite the ability of adoptively transferred T cells to reject tumor in these mice (39). In that model, however, both the tumor and some self tissues (i.e., T cells) expressing the Ag are MHC class II negative, requiring indirect effector mechanisms to mediate the antitumor activity as well as selected autoimmune injury. In contrast, in the current model both tumor and hepatocytes are MHC class I positive and therefore potentially directly sensitive to the lytic activity of the CD8+ CTL used in ACIT. In the gag-TG mice, liver parenchymal cells may partially resist injury attributable to expression of limited amounts of the counterreceptors, such as CD80 or CD86, that contribute to T cell-target avidity and provide costimulatory signals (40). Additionally, for some targets such as EBV-infected cells, susceptibility to the effector phase of the CD8+ response requires a higher density of the relevant viral Ags than stimulation of CD8+ responses (41).

Induction of tolerance can occur if APC take up self-Ag and then present it to T cells in a draining lymph node. This is analogous to some of the events involved in cross-priming, but presumably reflects failure to activate the APC with resultant delivery of tolerizing rather than activating signals (42, 43). Thus, the gag Ag may not be directly presented to T cells by hepatocytes, but rather by professional APC such as dendritic cells. Uptake of released Ag from uninjured liver cells or phagocytosis of apoptotic liver cells might not be expected to promote dendritic cell maturation. However, we attempted to promote autoreactivity by inducing inflammatory responses in the liver, followed by transfer of FMuLVgag-specific CTL. These experiments included infection of the livers of gag-TG mice with vaccinia or adenovirus, followed by injections of irradiated FBL as a stimulating Ag source, and IL-2 to promote survival and proliferation of the transferred CTL. No

FIGURE 7. Adoptively transferred FMuLVgag-reactive CTL can eradicate disseminated FBL tumor in gag-TG mice. B6 and gag-TG mice were inoculated on day 0 with $5 \times 10^6$ live FBL cells i.p. Absence of autoimmune liver injury following adoptive therapy of tumor in gag-TG mice. B6 and gag-TG mice were inoculated on day 0 with $5 \times 10^6$ live FBL cells i.p. Groups of mice were either left untreated or treated 5 days later with 180 mg/kg CY, followed in 6 h by transfer of a FMuLVgag-specific CD8+ CTL clone. Beginning on day 5, mice received daily injections of $10^6$ U of IL-2 i.p. daily. On day 12, livers from B6 and gag-TG mice were harvested, fixed in formaldehyde, paraffin-embedded, sectioned, stained with hematoxylin/eosin, and analyzed for inflammation and lymphocyte infiltration by a pathologist. Representative sections from B6 (A) and gag-TG (B) mice are shown (magnification, $\times25$).
evidence of injury to the liver was detectable following resolution of the acute infections (data not shown). This resistance of hepaticocytes to autoimmune damage may be a general phenomenon. In a transgenic model in which tolerance was induced by expression of the alloantigen H-2Kb in the liver, hepatocytes were very resistant to autoimmune injury (44), although CTL-mediated liver damage could be induced by infecting the mice with the liver-specific pathogen Listeria monocytogenes. The ability to ultimately induce autoimmune injury in this setting may be due to the tolerizing Ag being an allogeneic class I Ag, which cannot induce tolerance by cross-presentation via host APC and should be recognized by a broader host response.

Additional evidence suggesting liver cells may be insensitive targets for killing by T cells has been provided by studies of viral hepatitis. CD8+ T cells controlled viral replication in infected hepatocytes by release of inflammatory cytokines such as IFN-γ and TNF (45). Surprisingly, this biological activity was not accompanied by a cytopathic effect on hepatocytes. Thus, CD8+ T cells capable of killing targets can recognize liver cells and perform effector functions while not lysing the liver cells. The means by which hepatocytes may be protected from cytolysis are unclear, but similar mechanisms could be operative in our model.

The results of this murine study are consistent with observations following T cell therapy for human melanoma, in which T cells reactive with Ags expressed in both melanoma tumor cells and human melanocytes have been found to be therapeutically effective without mediating toxicity in most patients and inducing partial vitiligo in the remaining fraction (46). Our data validate the hypothesis that therapies based on T cell immunity to TAA overexpressed in tumor cells have the potential to provide significant therapeutic benefit. The ability to isolate and use such tumor-reactive T cells will likely be strongly dependent on the particular tissues that express the self-Ag, as well as the magnitude of normal expression. Further studies in relevant murine models such as the one presented here should help elucidate the principles necessary to facilitate development of this approach for human tumor therapy.

Acknowledgments
We thank Jean-Luc Darlix (Lyon, France) and Richard Palfi, William WA, for providing DNA constructs; Bruce Chesebro (Rocky Mountain Laboratories, Hamilton MT) for R187 Ab; Benjamin Jacobsen and Christopher Wilson (University of Washington, Seattle WA), for microinjection of the construct and generation of the gag-TG strain; Xiaoxia Tan and Jennifer Young for expert technical assistance; Laurence Cheng and Eric Huseby for helpful discussions; and Joanne Factor for her assistance in preparation of this manuscript.

References


