An Evaluation of the Potential to Use Tumor-associated Antigens as Targets for Antitumor T Cell Therapy Using Transgenic Mice Expressing a Retroviral Tumor Antigen in Normal Lymphoid Tissues

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Summary

A major obstacle to the development of T cell therapy for the treatment of human tumors has been the difficulty generating T cells specifically reactive with the tumor. Most of the characterized human tumor antigens have been classified as tumor associated, because of demonstrable expression at low levels in some normal cells, and thus have not been extensively studied as potential targets of a therapeutic immune response. However, the quantitative difference in expression of such antigens between the tumor and normal cells might permit the generation of antigen-specific T cells capable of selective antitumor and not autoimmune activity. To address this issue, transgenic (TG) mice were generated that expressed low levels of Friend murine leukemia virus (FMuLV) envelope protein in lymphoid cells under the control of an immunoglobulin promoter. This protein is expressed at high levels by a Friend virus–induced erythroleukemia of C57BL/6 (B6) origin, FBL, and has been shown to serve as an efficient tumor-specific rejection antigen in B6 mice. The *env*-TG mice were tolerant to envelope, as reflected by the failure to detect an envelope-specific response after in vivo priming and in vitro stimulation with preparations of FMuLV envelope. However, adoptively transferred envelope-specific T cells from immunized non-TG B6 mice mediated complete eradication of FBL tumor cells in TG mice, and did not induce detectable autoimmune damage to TG lymphoid tissues. The transferred immune cells were not permanently inactivated in the TG mice, since donor T cells responded to envelope after removal from the TG mice. The lack of autoimmune injury did not reflect inadequate expression of envelope by TG lymphocytes for recognition by T cells, since TG lymphocytes functioned effectively in vitro as stimulators for envelope-specific T cells. The results suggest that this and analogous strains of TG mice may prove useful for elucidating principles for the generation and therapeutic use of tumor-reactive T cells specific for tumor-associated antigens.

Effector T cells have been demonstrated to mediate protective and therapeutic immune responses against tumors in both animals and humans. The generation of such responses requires that the tumor cells express an antigen recognizable by host peripheral T cells. The ideal tumor antigens are immunogenic proteins uniquely expressed on neoplastic cells. Although there are many examples of such antigens in experimental animal tumors as well as more limited examples in human tumors, most of the human tumor antigens that have been characterized are also detected at low levels in some normal cells (1, 2). Efforts to evaluate such antigens as targets for the generation of therapeutic antitumor T cell responses have been limited largely due to two issues: (*a*) the host is likely to be tolerant to these tumor-associated antigens (TAA)[1] since they are normal self-proteins such as differentiation antigens; and (*b*) even if methods to break tolerance and elicit host T cell responses to the antigen could be

[1] *Abbreviations used in this paper:* CY, cyclophosphamide; Eμ, heavy chain μ enhancer; FBL, Friend virus–induced leukemia of B6 origin; FMuLV, Friend murine leukemia virus; hGH, human growth hormone; Pμ, heavy chain μ Vh promoter; SA, splice acceptor site; SD, splice donor site; TAA, tumor-associated antigen; TG, transgenic.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/06/1681/10 $2.00
Volume 177 June 1993 1681–1690

identified, such effector cells may not only have potential antitumor activity but also the capability of mediating autoimmune damage to the normal tissues expressing the antigen.

Advances in our understanding of the basis and maintenance of tolerance to self-antigens have made it possible to reexamine such possible target antigens. Studies in transgenic (TG) mice, in which genes have been introduced into the germline and consequently encode new "self-proteins," have revealed that the interaction between host T cells and self-antigens can result in several distinct outcomes. Intrathymic expression or presentation of self-antigens usually leads to deletion of self-reactive T cells (3–5). By contrast, self-antigens expressed only in peripheral (extrathymic) tissues often induce tolerance by nondeletional mechanisms, such as clonal anergy (6, 7), and/or downregulation of the TCR (8). Despite apparent tolerance in vivo, T cells specific for some extrathymic self-antigens can be activated under appropriate conditions in vitro (9–11) and in vivo (12, 13). Moreover, some extrathymic self-antigens may be expressed in such limited amounts or found in normal tissues expressing a sufficiently low density of MHC molecules that the antigens fail to induce tolerance or serve as targets for autoimmune reactivity (14, 15). These findings suggest that some TAA may be capable of serving as targets for antitumor T cell therapy, a perception further supported by the recent demonstration that tumor-specific T cells isolated from a patient with melanoma are specific for a protein encoded by a nonmutated gene (16).

Our laboratory has previously described and extensively characterized an adoptive T cell therapy model for the treatment of a disseminated Friend virus–induced leukemia of B6 origin, FBL (17). FBL expresses Friend murine leukemia virus (FMuLV) *env*- and *gag*-encoded tumors antigens (18, 19), and FBL-reactive $CD8^+$ CTL have been shown to predominantly recognize FMuLV *gag*-encoded proteins while FBL-reactive $CD4^+$ Th cells recognize FMuLV *env*-encoded antigens (19). Each T cell subset, independent of the other, can mediate complete eradication of disseminated FBL tumor cells in adoptive T cell therapy of B6 mice (18, 20, 21).

To investigate the potential for using TAA as targets for adoptive T cell therapy of tumors, lines of B6 transgenic mice expressing FMuLV genes under the control of various tissue-specific promoters have been generated. These TG animals provide a model, with the FBL tumor, to evaluate: (*a*) the basis of host T cell tolerance to TAA; (*b*) the requirements for breaking such tolerance to induce a host T cell response to TAA; and (*c*) the consequences of inducing such a T cell response with regard to both tumor eradication and autoimmune injury. This paper is the initial description and characterization of a TG mouse line expressing low levels of FMuLV envelope in the lymphoid tissues, under the control of the mouse Ig heavy chain μ enhancer ($E\mu$) and Vh promoter ($P\mu$) element. Our results show that FMuLV envelope antigen expression in TG lymphoid tissues induces T cell tolerance, in that host T cells cannot be readily primed to FMuLV envelopes in vivo. In contrast, FMuLV envelope–specific primed T cells derived from non-TG mice are not inactivated after transfer into *env*-TG mice, and mediate eradication of envelope-expressing FBL tumor cells without causing detectable autoimmune destruction of the lymphoid tissue expressing low levels of FMuLV envelope. The possible mechanisms are discussed.

Materials and Methods

Mice. C57BL/6 (H-2^b, Thy-1.2, denoted B6) mice and breeding pairs of congenic B6.PL (74NS) (H-2^b, Thy-1.1, denoted B6/Thy-1.1) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Cell Lines and Viruses. FBL expresses FMuLV *env*- and *gag*-encoded products and class I but not class II H-2^b MHC molecules (18, 19). FMuLV virions, live recombinant vaccinia virus expressing the FMuLV *env* gene (22), and vaccinia virus were kindly provided by Dr. Bruce Chesebro (Rocky Mountain Laboratories, Hamilton, MT).

Gene Construction. The pIg-ENV-human growth hormone (hGH) fusion gene (Fig. 1) was constructed using a multi-step procedure. A 2.2-kb EcoRI-SstI fragment of plasmid PICμPRE8 (W. Kindsvogel, ZymoGenetics, Seattle, WA), containing the combined $E\mu$ and $P\mu$ sequences derived from the mouse Ig heavy chain gene (23, 24), was cloned into PUC19 to generate plasmid PJH1. A 2.2-kb XbaI-PstI fragment of FMuLV clone 57, containing the entire FMuLV *env* gene (25), was subcloned into PUC18 to generate plasmid PWK4. A 2.2-kb XbaI-HindIII fragment, containing the FMuLV *env* gene, was then isolated from PWK4 and inserted into the XbaI and HindIII sites of PJH1, immediately downstream of the $E\mu/P\mu$ segment, to generate plasmid PJH2. Finally, a 2.1-kb BamHI-HindIII fragment containing the hGH gene sequence extending from the BamHI site in the first exon to the EcoRI site after the poly(A) addition signal (26) was isolated from plasmid P107 (27) and inserted with a synthetic linker into the HindIII site of PJH2. The final plasmid (pIg-ENV-hGH) contains the $E\mu/P\mu$ regulatory elements, the FMuLV *env* gene and hGH gene oriented 5′ to 3′ (Fig. 1). A 6.5-kb EcoRI fragment of pIg-ENV-hGH was isolated for microinjection.

The pIg-ENV-3′LTR fusion gene was constructed in multiple steps. Complimentary oligonucleotides were synthesized to contain bases 170–215 of FMuLV clone 57, which includes the splice donor site (SD) for FMuLV at position 206 (EMBL/GenBank Nucleotide Sequence Databases), and new SalI and SphI sites at the 5′ and 3′ ends, respectively. This synthetic oligonucleotide was joined at the 5′ SphI site of a 4.1-kb SphI-HindIII fragment of FMuLV clone 57, which includes the splice acceptor site (SA), entire *env* coding region, and 3′ LTR sequence of FMuLV clone 57 (25, 28), and then cloned into PUC 18 at SalI-HindIII sites to generate plasmid PJH11. Finally, a 2.2-kb EcoRI-SalI fragment of PJH1, which contains the combined $E\mu/P\mu$ regulatory sequence, was inserted between the EcoRI and SphI sites of pJH11, immediately upstream of the FMuLV *env* gene. The final plasmid (pIg-ENV-3′LTR) contains the $E\mu/P\mu$ control elements and the FMuLV env-3′LTR gene (Fig. 1). A 6.3-kb EcoRI-HindIII fragment of pIg-ENV-3′LTR was used for microinjection.

Generation of TG Mice. Transgenic mice were generated using standard methods (29). Briefly, purified linear DNA (2 ng/μl) was injected into pronuclei of B6 zygotes, and viable zygotes were implanted into the oviducts of pseudopregnant Swiss Webster female mice. The presence of integrated pIg-ENV-3′LTR in pups was determined by hybridization of a Southern blot of PstI-digested tail DNA to a ^{32}P-labeled 870-bp BamHI-PstI fragment of the FMuLV *env* gene probe (25). Mice containing the pIg-ENV-hGH trans-

gene were determined by dot blot analysis (30) using a ^{32}P-labeled 1.3-kb PstI fragment of hGH probe (26). Transgene-positive lines were bred for at least three generations to document stable transmission of the transgene before further analysis.

RNA Blot Analysis. Total cellular RNA from various tissues was isolated for analysis of gene expression by the guandinium-isothiocyanate/CsCl method (31). The RNA samples were subjected to electrophoresis in 1% agarose-formaldehyde gels, transferred to nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH), and hybridized to an 870-bp BamHI-PstI fragment of the FMuLV *env* gene (25) labeled with ^{32}P by random priming (32). For selected studies, the RNA was obtained from enriched B cell populations purified from spleen cells by panning on dishes coated with rabbit anti-mouse Ig (33), or from enriched T cell populations purified from the nonadherent cells removed from the anti-Ig plates by passage through nylon wool columns (34).

Purification of FMuLV Envelope Proteins Expressed in TG Lymphocytes. Affinity-purified goat antibody (IgG) against the gp70 envelope of Rauscher murine leukemia virus (RMuLV) and crossreactive with FMuLV (NCI/BCB Repository, Bethesda, MD) was oxidized and coupled to an Affi-Gel Hz hydrazide gel column (Bio-Rad Laboratories, Richmond, CA) (35). Spleen cells from pIg-ENV TG mice or non-TG littermates were resuspended in lysis buffer containing 20 mM Hepes (pH 7.3), 1 mM EDTA, 50 mM NaCl, and 0.4% NP40, homogenized, and centrifuged at 500 *g* for 10 min. The whole cell lysates were applied to the antibody affinity column and washed through with 0.5 M NaCl in 0.1 M Tris buffer, pH 8.0. The absorbed FMuLV envelope proteins were subsequently eluted with 3 M potassium thiocyanate in PBS.

Western Blot. The protein samples eluted from the affinity columns and unpurified splenocyte lysates were fractionated by reducing SDS-PAGE (10%) and electroblotted onto nitrocellulose. The blot was blocked 1 h at room temperature with 5% nonfat milk in TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5), and probed for 2 h with goat antiserum to gp70 diluted 1:300 in TTBS. The blot was subsequently washed three times for 10 min each in TTBS, and incubated for 1 h with an ^{125}I-labeled affinity-purified rabbit anti-goat Ig (2×10^5 cpm/ml) in TTBS. The washed filter was exposed to Kodak X-omat XAR-5 film at −70°C with intensifying screens.

Immunization. 6–8-wk-old mice were inoculated via tail scratch with 10^7 PFU of recombinant vaccinia-FMuLV *env* (*vac-env*) virus or vaccinia (*vac*) virus. After a delay of at least 3 wk, spleen cells from primed mice were obtained for in vitro analysis. For adoptive transfer experiments, donor mice were boosted with a second dose of virus 3 wk after the initial priming.

In Vitro T Cell Proliferation Assay and IL-2 Assay. T cell proliferative responses to FMuLV envelope were evaluated as described (36). Briefly, in vivo primed spleen responder cells were cultured with either irradiated stimulator cells or UV-inactivated virus in 96-well flat-bottomed plates. The origin, preparation, and number of responder cells and stimulator cells, as well as total length of the culture period, are indicated in each table or figure legend. [^{3}H]TdR (20 mCi/mmol; New England Nuclear, Boston, MA), 1.0 μCi/well, was added for the final 24 h of culture. The IL-2 activity present in cell-free culture supernatants was determined by measuring [^{3}H]TdR uptake by CTLL, an IL-2-dependent T cell line (37).

Adoptive Cell Transfer. Immune splenocytes were obtained from B6/Thy-1.1 mice immunized with *vac-env* or *vac* as described above. 10^8 spleen cells were injected intraperitoneally into pIg-ENV TG mice and non-TG control mice. To promote in vivo proliferation of transferred immune T cells, irradiated FBL tumor cells (10^7) and human rIL-2 (5,000 U/d for 6 d) were also injected intraperitoneally into selected groups of recipient mice (38). Animals were killed 2, 4, and 12 wk later, and the spleens, mesenteric lymph nodes, and thymuses were removed and processed for histologic examination.

Histology. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin/eosin. Veterinarian pathologists reviewed the slides to evaluate the lymphoid tissues for pathologic injury.

Analysis of Peripheral Blood T and B Cells on the FACS®. Single cell suspensions of RBC-lysed peripheral blood cells were labeled with either FITC-conjugated monoclonal anti-Thy-1.2 (Becton Dickinson Monoclonal Center, Sunnyvale, CA) or FITC-conjugated goat anti-mouse Ig serum (Tago Inc., Burlington, CA) and analyzed on the FACS® (39).

Adoptive Chemoimmunotherapy (ACIT). This model, as previously described (17, 20), consists of treating mice bearing established disseminated FBL leukemia with a combination of nonlethal noncurative chemotherapy and adoptively transferred immune cells. Briefly, on day 0, pIg-ENV TG mice were inoculated intraperitoneally with 5×10^6 viable FBL leukemia cells. By day 5, after tumor cells had disseminated, mice were treated with 180 mg/kg cyclophosphamide (CY) intraperitoneally, followed in 5 h by adoptively transferred donor cells. Donor splenocytes obtained from B6 mice primed with *vac-env* or *vac* as described above were enriched for T cells by passage through a nylon wool column. Previous studies have demonstrated that therapy on day 5 with immune T cells alone has no apparent antitumor effect, in part due to the large tumor burden (17, 40), but that combined treatment with CY plus immune T cells can mediate complete tumor elimination (17). The major contribution of CY in this model appears to be a reduction of the tumor burden to a size small enough to permit effective expression of the antitumor activity of transferred immune T cells (17).

Results

Generation of TG Mice and Expression of the FMuLV env Transgene in Lymphoid Tissues. To generate TG mice expressing the FMuLV *env* gene in the lymphoid lineage, two transgene construction strategies were used. The first approach was to ligate a 2.2-kb mouse Eμ/Pμ segment to a 4.1-kb fragment of FMuLV containing the SD, SA, *env* gene, and 3′LTR sequences (see Materials and Methods) to generate the pIg-ENV-3′LTR fusion gene (Fig. 1). This construct resulted in expression of the FMuLV *env* gene after transfection in vitro into SP2/0, a mouse myeloma cell line (41). However, in seven TG founder animals derived from microinjection of the 6.3-kb EcoRI-HindIII fragment of pIg-ENV-3′LTR into B6 zygotes, no transgene expression was detected. Since the presence of introns can enhance gene expression in TG mice (42), we adopted an alternative construction strategy. The 2.2-kb FMuLV *env* gene (25) was inserted downstream of the 2.2-kb mouse Eμ/Pμ regulatory segment and ligated to the 2.1-kb intron-containing hGH gene (Fig. 1, *pIg-ENV-hGH*), such that the hGH sequences provide a polyadenylation signal and intronic sequences to enhance transgene expression, but do not produce hGH protein (42, 43). After

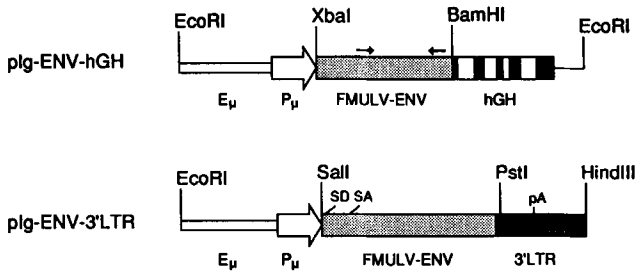

Figure 1. Map of the transgene constructs. The pIg-ENV-hGH construct contains a 2.2-kb FMuLV *env* gene (*stippled bar*) located downstream from a 2.2-kb combined μ enhancer (Eμ, *open bar*)/Vh promoter (Pμ, *arrow*) fragment, derived from the mouse Ig heavy chain gene. This gene was fused into the first exon of a 2.1-kb genomic hGH gene (exons, *filled boxes*; introns, *open boxes*). The pIg-ENV-3'LTR construct contains a 2.2-kb combined Eμ/Pμ fragment fused to a 4.1-kb segment including the splice donor site (*SD*), splice acceptor site (*SA*), *env* gene (*light stippled bar*), and 3' LTR sequence (*dark stippled bar*) of FMuLV clone 57. The position of the probe used to analyze gene expression, an 0.87-kb fragment of the FMuLV *env* gene, is located between the two solid arrows.

microinjection of the 6.5-kb linearized pIg-ENV-hGH hybrid DNA fragment into pronuclei of B6 mouse zygotes, 30 pups were born, with two containing the integrated pIg-ENV-hGH transgene. Both founders (lines 3164 and 3170) passed the transgene to their progeny, but only line 3164 mice transcribed the FMuLV *env* transgene mRNA and expressed protein in lymphoid tissues (Fig. 2).

Total RNA from various tissues and cells from line 3164 was isolated and analyzed by blot hybridization using a ^{32}P-labeled 870-bp BamHI-PstI fragment of the FMuLV *env* gene (25) as the probe (Fig. 2). The transgene-encoded FMuLV *env* transcript was found in lymphoid cells (T and B populations) and organs (spleen and thymus) of pIg-ENV TG mice,

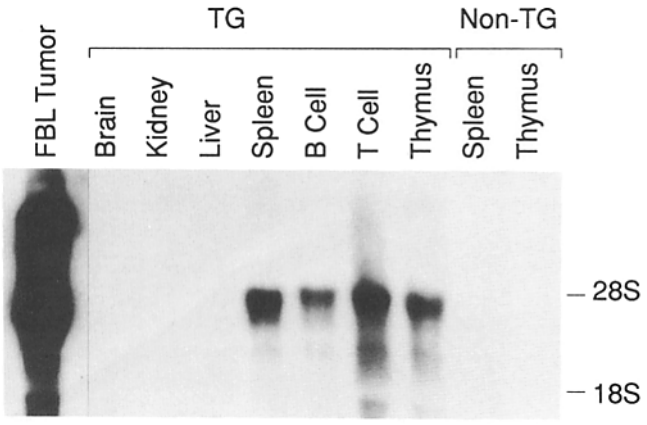

Figure 2. Specific expression of FMuLV *env* mRNA in lymphoid tissues from pIg-ENV TG mice. 2 μg of total RNA from cultured FBL tumor cells and 20 μg of total cellular RNA from the indicated tissues and lymphocytes of a pIg-ENV TG mouse (line 3164) and a non-TG littermate were fractionated by electrophoresis in a 1% agarose-formaldehyde gel, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled 870-bp BamHI-PstI fragment of the FMuLV *env* gene. The migration of eukaryotic ribosomal RNAs, as markers, is indicated.

and was expressed at much lower levels than in the FBL tumor. The transcript was not detected in brain, kidney, or liver. Efforts to identify the transgene-encoded FMuLV envelope protein in lymphoid cells by immunofluorescence, immunocytochemistry, and Western blotting techniques were unsuccessful, although these techniques all detected the protein in the FBL tumor. Since conventional antibody assays usually require a minimum of several thousand to one hundred thousand protein molecules for detection (44, 45), a more sensitive method for detecting protein was explored. Potential FMuLV protein present in a TG spleen cell lysate was purified by immunoaffinity chromatography, a method that can achieve 1,000–10,000-fold protein purification (46). The protein attached to the column was eluted and tested for the presence of FMuLV envelope by Western blot analysis (Fig. 3). Using this strategy, the transgene-derived FMuLV envelope precursor protein gp85^{env} and the processed glycosylated protein gp70^{env} were detected from TG spleen cells. As a negative control, non-TG littermates derived from matings of line 3164 were studied, and no mRNA or protein was detected (Figs. 2 and 3).

To determine if the low level of FMuLV *env* expression in TG lymphocytes is sufficient for recognition by T cells, the ability of FMuLV envelope–specific T cells to proliferate in response to irradiated FMuLV *env*$^+$ TG lymphocyte stimulator cells was assessed (Fig. 4). Irradiated spleen cells from non-TG B6 mice were unable to trigger proliferation of either the CD4$^+$ T cell clone B10, a previously described gp70-specific clone of B6 origin (47), or immune spleen T cells from B6 mice primed with a vaccinia-FMuLV *env* recombinant virus (*vac-env*). In contrast, irradiated TG FMuLV *env*$^+$ spleen lymphocytes, as well as two other FMuLV *env*$^+$ stimulators, FBL cells and UV-inactivated FMuLV virus, stimulated both types of FMuLV envelope–specific T cells. Thus, the amount of *env* expressed in TG cells can, under appropriate conditions, be recognized by envelope-specific T cells and stimulate T cell responses.

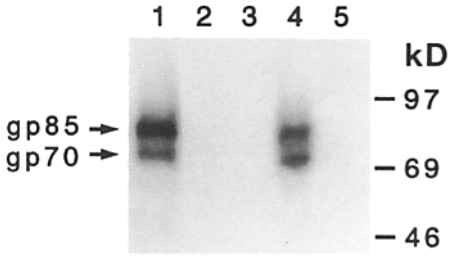

Figure 3. Expression of FMuLV envelope proteins in lymphocytes of TG mice. Spleens were obtained from 6-wk-old pIg-ENV TG mice and non-TG littermates. The whole spleen cell lysates were prepared and purified for FMuLV envelope proteins by immunoaffinity chromatography. The protein samples were separated by reducing SDS-PAGE (10%), electroblotted onto nitrocellulose, and probed using affinity-purified goat antiserum to gp^{70} envelope and a ^{125}I-labeled second-step rabbit anti–goat antibody (see Materials and Methods). Lane *1*, 100 μg FBL cell lysate; lane *2*, 100 μg spleen cell lysate from non-TG littermates; lane *3*, 10 μg affinity-purified proteins from spleen cell lysate of non-TG littermates; lane *4*, 10 μg affinity-purified proteins from spleen cell lysate of TG mice; lane *5*, 100 μg spleen cell lysate from TG mice. Mobilities of prestained molecular mass standards are indicated at the right.

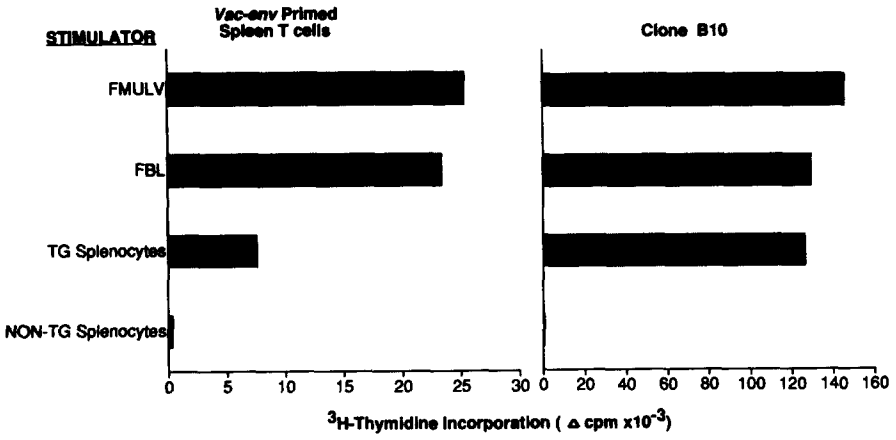

Figure 4. Ability of FMuLV envelope-expressing TG lymphocytes to stimulate proliferative responses in FMuLV envelope-reactive T cells. Spleen cells were obtained from B6 mice primed with *vac-env* and enriched for T cells by passage over nylon wool columns. 10^5 *vac-env*-primed splenic T cells or 5×10^4 B10 cells, an envelope-specific $CD4^+$ T cell clone, were cultured for 3 d with 5×10^5 irradiated B6 splenocytes (1,700 rad) as APC plus indicated stimulators, including 2×10^5 irradiated TG or non-TG spleen cells (1,700 rad), 2×10^4 irradiated FBL cells (10,000 rad), and 5 μg/ml of UV-inactivated FMuLV virion (*FMuLV*). [^{3}H]TdR (1.0 μCi/well) was added during the final 24 h of culture. Data presented were calculated as described in Table 1.

T Cell Tolerance to FMuLV Envelope Antigen in pIg-ENV TG Mice. To evaluate T cell responsiveness in pIg-ENV TG mice to FMuLV envelope antigen, groups of TG mice and non-TG littermates were inoculated via tail scratch with *vac-env*. Spleen cells were obtained a minimum of 3 wk after immunization, and the response to FMuLV envelope was determined by in vitro T cell proliferation and IL-2 production assays (Table 1). As expected, T cells from *vac-env*-primed non-TG mice responded to stimulation with FBL tumor cells or UV-inactivated FMuLV virus both by proliferating and producing IL-2. In contrast, T cells from *vac-env*-primed TG mice exhibited no response to either preparation of FMuLV envelope antigen. TG T cells primed with *vac-env* responded normally to vaccinia virus, affirming that such T cells were infected with vaccinia virus and were competent to respond.

Table 1. *FMuLV Envelope-specific T Cell Proliferation and IL-2 Production*

Mixed leukocyte tumor culture*		T cell proliferation	IL-2 production (CTLL)
Responder	Stimulator		
		Δcpm‡	*Δcpm*§
TG *αvac-env*	$(FBL)_x$	439	152
	FMuLV	534	398
	Vac	29,461	20,952
B6 *αvac-env*	$(FBL)_x$	19,493	8,906
	FMuLV	21,002	14,748
	Vac	32,145	31,140
B6	$(FBL)_x$	591	72
	FMuLV	664	62
	Vac	720	226

* Responder spleen cells were obtained from pIg-ENV TG mice or non-TG B6 littermates previously immunized with *vac-env* or left unprimed. To assay the T cell proliferative response, responder cells (5×10^5/well) were cultured for 3 d with 10^4 irradiated $(FBL)_x$ or $(B6)_x$ spleen cells, 5 μg/ml UV-inactivated FMuLV virions (FMuLV), or vaccinia virus (*vac*) at a 1:1 multiplicity of infection. 1.0 μCi/well [^{3}H]TdR was added for the final 24 h of culture. To assay IL-2 production, responder $CD4^+$ T cells were enriched from spleen cells by negative selection (19), and 2×10^6 $CD4^+$ responder T cells were cultured with the indicated stimulators at the same responder/stimulator ratio used in the T cell proliferation assay. After 40 h of incubation, cell-free supernatants were collected and assayed for IL-2 activity by measuring CTLL proliferation (37).

‡ Δcpm, are the mean cpm of triplicate cultures with antigen stimulation minus the mean cpm of triplicate cultures without antigen stimulation.

§ Data represents the IL-2 activity present in a 1:4 dilution of the culture supernatant. Δcpm were calculated as the proliferative response of CTLL cells, an IL-2 indicator line, induced by supernatants from antigen-stimulated cultures minus the response with supernatants from unstimulated cultures. A representative experiment is presented.

Responsiveness to envelope was also assessed by examining resistance of TG and non-TG mice to challenge with FBL tumor. Unimmunized TG mice and non-TG littermates all died within 20 d of challenge with 5×10^5 viable FBL tumor cells (Table 2). Prior immunization with *vac-env* protected 14 of 16 non-TG mice from challenge with this tumor, as demonstrated by rejection of the viable tumor cells and long-term survival. In contrast, *vac-env*-immunized TG mice remained susceptible to FBL tumor challenge, with all mice dying of progressive tumor growth by day 20. Moreover, *vac-env* immunization did not delay tumor progression and time of death in TG mice. The results of these in vitro and in vivo assays suggest that T cells in TG mice are tolerant to the FMuLV envelope antigen.

Adoptive Transfer of FMuLV Envelope-immune T Cells into pIg-ENV TG Mice Does Not Cause Detectable Autoimmune Injury in FMuLV env-expressing Lymphoid Tissues. TG mice expressing FMuLV *env* provide a model for evaluating the consequences of adoptive therapy with tumor-reactive T cells that can potentially recognize normal tissues. Thus, FMuLV envelope-immune spleen cells obtained from congeneic B6/Thy-1.1 donor mice primed with *vac-env* were injected into pIg-ENV TG mice. To promote the proliferation of transferred FMuLV envelope-specific donor T cells in recipients, irradiated FBL tumor cells and/or rIL-2 were also injected into selected groups of recipient mice (Table 3). Mice were killed at multiple time points after cell transfer, and evaluated for immune-mediated injury. No detectable histopathologic changes were observed in the TG lymphoid organs, and no decrease in the number of peripheral blood T or B lym-

Table 2. *Failure to Induce Protective Immunity against FBL Tumor Challenge in pIg-ENV TG Mice after Immunization with vac-env*

Mice	No./group	Immunization‡	Tumor challenge§	Percent survival*		
				15 d	20 d	60 d
TG	16	*vac-env*	FBL	38	0	0
	15	*vac*	FBL	40	0	0
	15	None	FBL	46	0	0
B6	16	*vac-env*	FBL	100	88	88
	16	*vac*	FBL	43	0	0
	16	None	FBL	38	0	0

* The cumulative data of two independent experiments are provided, with percent survival recorded at 15, 20, and 60 d after tumor challenge.
‡ pIg-ENV TG mice and non-TG B6 littermates were immunized via tail scratch with *vac-env* or *vac*, or left unprimed as described in Materials and Methods.
§ Mice were challenged intraperitoneally with 5×10^5 viable FBL leukemia cells 4 wk after the immunization.

phocytes was detected. The TG recipients of transferred immune T cells appeared healthy, gained weight normally, and did not exhibit runting, the most common clinical features of GVHD (48). These data suggest that the low levels of FMuLV envelope expressed in TG lymphoid tissues may not be capable of stimulating envelope-reactive T cells in vivo despite the fact that the TG cells can stimulate such T cells under appropriate in vitro conditions.

Table 3. *Effect of Adoptive Transfer of FMuLV Envelope-immune T Cells into pIg-ENV TG Mice on the TG Lymphoid Compartment*

Donor cells§	Recipients	Additional treatment*		Histology of lymphoid tissues‖	Blood lymphocyte counts‡			
					T cells		B Cells	
		FBL_x	rIL-2		Day 0	Dat 28	Day 0	Day 28
					$\times 10^3/mm^3$			
vac-env-primed B6/Thy-1.1 spleen T cells	TG	–	–	Normal	5.05 ± 0.31	5.21 ± 0.25	1.91 ± 0.12	2.13 ± 0.16
		+	–	Normal	4.95 ± 0.34	7.31 ± 0.51	1.85 ± 0.14	3.01 ± 0.25
		–	+	Normal	5.10 ± 0.41	5.51 ± 0.45	1.92 ± 0.13	2.21 ± 0.24
		+	+	Normal	4.91 ± 0.25	8.23 ± 0.75	2.01 ± 0.17	3.51 ± 0.31
	B6	–	–	Normal	4.85 ± 0.37	5.02 ± 0.35	1.93 ± 0.14	1.90 ± 0.11
		+	–	Normal	5.07 ± 0.29	8.11 ± 0.85	2.07 ± 0.17	3.62 ± 0.37
		–	+	Normal	4.98 ± 0.41	5.24 ± 0.36	1.87 ± 0.15	2.07 ± 0.16
		+	+	Normal	5.15 ± 0.32	9.76 ± 0.98	1.98 ± 0.18	4.05 ± 0.38
vac-primed B6/Thy-1.1 spleen T cells	TG	–	–	Normal	4.82 ± 0.40	4.97 ± 0.31	2.10 ± 0.12	2.15 ± 0.17
	B6	–	–	Normal	5.21 ± 0.39	5.18 ± 0.43	1.79 ± 0.19	2.01 ± 0.14

* The indicated recipients were injected intraperitoneally with 10^7 irradiated (12,000 rad) FBL cells and/or 5,000 U/d for 6 d of human rIL-2.
‡ Venous blood samples were obtained from recipient mice before (day 0) and after (day 28) adoptive transfer of B6/Thy-1.1 donor T cells. Total white blood cells were counted in a hemocytometer, the RBC were lysed, and the percent host peripheral blood T and B cells subsequently enumerated on a FACS® with the use of fluoresceinated monoclonal anti-Thy-1.2 or goat anti-mouse Ig serum.
§ Donor spleen cells were obtained from B6/Thy-1.1 mice immunized with either *vac-env* or *vac* as described in Materials and Methods. Unfractionated spleen cells (10^8) were injected intraperitoneally into indicated pIg-ENV TG mice or non-TG B6 littermates. The data from two experiments containing a total of four mice/group are presented.
‖ Animals were killed 2, 4, and 12 wk after transfer of donor cells, and the spleens, mesenteric lymph nodes, and thymuses were removed for histologic examination. Normal histology means that no pathologic changes in cell number, distribution, or architecture were observed.

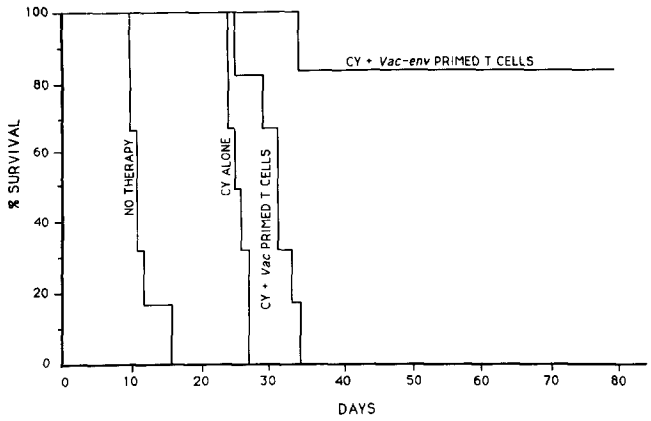

Figure 5. Efficacy of transferred T cells immune to FMuLV envelope in the therapy of disseminated FBL leukemia in pIg-ENV TG hosts. Immune spleen cells were obtained from B6 mice immunized with *vac-env* or *vac* and were enriched for T cells by passage over nylon wool columns. pIg-ENV TG ice (six mice/group) were inoculated intraperitoneally with 5×10^6 viable FBL tumor cells on day 0 and were left untreated (*NO THERAPY*), treated on day 5 with 180 mg/kg CY (*CY ALONE*), CY plus 5×10^7 *vac*-primed B6 T cells, or CY plus 5×10^7 *vac-env*-primed B6 T cells.

Expression of FMuLV env in TG Lymphoid Tissue Does Not Interfere with Activity of Transferred FMuLV Envelope–immune T Cells in pIg-ENV TG Recipients. Previous studies in H-2K^b TG mice showed that expression of the H-2K^b transgene in peripheral tissue could tolerize adoptively transferred H-2K^b-reactive T cells (49), suggesting that even fully mature peripheral T cells can be the targets for tolerance induction. Such tolerance induction would explain the failure to observe autoimmune tissue injury in the adoptive transfer studies described above. This issue was further evaluated in an adoptive therapy model for the treatment of disseminated leukemia with a combination of chemotherapy and immune T cells (17). In this model, complete eradication of the tumor is dependent on the long-term persistence and function of transferred tumor-specific T cells (39) and, consequently, in vivo tolerance induction would be expected to interfere with the therapeutic antitumor activity of T cells. TG mice were inoculated with 5×10^6 viable FBL on day 0 and, if given no therapy, had a median survival of 12 d (Fig. 5). Treatment with CY alone on day 5 prolonged the median survival to day 25, but all mice still died of progressive tumor growth. As an adjunct to CY, adoptive transfer of 5×10^7 splenic T cells from *vac*-primed B6 mice had no significant effect on survival. In contrast, therapy with CY plus 5×10^7 *vac-env*-primed splenic T cells prolonged survival and cured >80% of mice. Moreover, histologic analysis of these cured mice revealed no evidence of autoimmune injury in the lymphoid tissue. Thus, transferred FMuLV envelope–immune T cells can maintain antitumor activity and mediate eradication of FMuLV *env*$^+$ FBL cells in TG hosts, despite persistent exposure to FMuLV envelope expressed in lymphoid tissues.

The ability of transferred immune T cells to retain activity in TG recipients was examined in an additional model. Immune spleen cells from B6 mice primed with *vac-env* were transferred into TG animals. The recipients were then boosted with *vac-env* and, 4 wk later, spleen cells were obtained and assessed for T cell reactivity to FMuLV envelope (Table 4). Spleen cells recovered from TG recipients of *vac-env*-primed donor T cells demonstrated proliferative responses to FBL, FMuLV virus, and vaccinia. In contrast, spleen cells from TG mice that did not receive transferred *env*-reactive donor T cells responded to vaccinia, but did not respond to FBL or FMuLV. The cumulative results demonstrate that FMuLV envelope–specific immune donor T cells are not inactivated when transferred into pIg-ENV TG mice, retain responsiveness to an *env*-expressing tumor, and do not induce readily demonstrable autoimmune injury in lymphoid tissues expressing low levels of the transgene product.

Discussion

In the present study we have described a TG mouse model useful for addressing some of the issues related to the use of tumor-associated antigens not uniquely expressed on transformed cells as targets for adoptive T cell therapy of human tumors. The model uses a retrovirally transformed leukemia FBL that expresses immunogenic retrovirus-encoded antigens, and TG mice that express one of these proteins, FMuLV envelope, under the control of a lymphoid-specific promoter. Our results demonstrate that expression of the FBL tumor an-

Table 4. *FMuLV Envelope-specific Proliferative Response of Donor T Cells Recovered from pIg-ENV TG Recipients*

	In vivo pretreatment*		T cell proliferative response‡		
Responder	Adoptive transfer	Immunization	FBL	FMuLV	*Vac*
				Δcpm	
TG	*vav-env*-primed B6 spleen cells	*vac-env*	6,062	5,203	52,206
TG	Medium	*vac-env*	524	−423	51,841

* PIg-ENV TG mice injected intraperitoneally with medium or 10^8 spleen cells from B6 mice previously primed with *vac-env*, and then immunized via tail scratch with *vac-env*.

‡ 4 wk after in vivo pretreatment, spleen cells were obtained and assessed for T cell proliferative responses, as described in Table 1. A representative experiment is presented.

tigen in normal cells of the same lymphohematopoietic lineage as the tumor, but at substantially lower levels than the tumor, can induce T cell tolerance, as reflected by the inability to elicit an envelope-specific T cell response after immunization of TG mice with a recombinant vaccinia virus expressing the envelope antigen. However, the expression of low levels of the envelope in TG mice does not interfere with the therapeutic efficacy of adoptively transferred envelope-specific T cells, and such cells can mediate tumor rejection without inducing autoimmune injury in TG mice.

The TG mice were constructed by introducing the FMuLV *env* gene into B6 mice under the control of the Ig $E\mu/P\mu$ promoter. This resulted in selective expression of *env* in T cells as well as B cells, as predicted from other TG mouse strains containing genes controlled by the Ig promoter (50). Although our results demonstrate that the TG mice are tolerant to the transgene-encoded FMuLV envelope, the basis for such tolerance in these mice has not been fully clarified. Analysis in normal B6 mice of TCR $V\beta$ gene usage by T cells responsive to FMuLV envelope has failed to detect a dominant $V\beta$ gene (data not shown), and thus it is not possible to determine if a distinct envelope-reactive T cell population has been clonally deleted. The expression of envelope in T and B cells assures that this antigen is present in the thymus during thymic ontogeny, but does not a priori indicate that clonal deletion will occur, since neither cell type has been definitively shown to mediate negative selection in the thymus.

Alternative nondeletional peripheral mechanisms of tolerance include induction of peripheral anergy, such as by improper triggering and/or downregulation of the TCR, or active suppression (6–8, 51). This latter mechanism of suppression seems improbable, since adoptively transferred envelope-reactive T cells were effective in tumor therapy in TG mice. Moreover, the demonstration that transferred envelope-reactive T cells can be recovered from TG mice with retention of function suggests that, if peripheral anergy to envelope is being induced in TG mice, the peripheral mechanism is only operative to prevent priming of naive T cells and is not operative with T cells previously primed and/or activated in vitro. This is entirely consistent with the known more rigid requirements for inducing a primary rather than activating a secondary T cell response (52). Definitive determination of the mechanism responsible for tolerance induction and maintenance will likely require the construction of TG mice expressing an envelope-specific TCR gene, such as could be derived from the B10 T cell clone. Such TCR-TG mice could then be mated to the *env*-TG mice, and the fate of T cells through the thymus and periphery could be assessed.

Adoptive transfer of FMuLV envelope–immune T cells into pIg-ENV TG mice did not result in any apparent autoimmune disease, as reflected by the absences of pathological changes in TG lymphoid tissues, decline in the number of host-circulating T and B cells despite the expression of envelope by these cells, or clinical features of GVHD. This does not reflect clonal inactivation of the transferred T cells, since such cells mediated in vivo tumor reactivity and could be recovered with retention of activity after transfer into TG mice. Previous studies from our laboratory have demonstrated that FMuLV envelope selectively induces in B6 mice only an MHC class II–restricted $CD4^+$ T cell response, and consequently no MHC class I–restricted $CD8^+$ cytolytic T cell response to envelope is detectable after immunization with either an envelope-expressing tumor or a recombinant *vac-env* (19). Thus, the lack of autoimmune tissue destruction could reflect a requirement for both $CD4^+$ and $CD8^+$ T cells for autoimmune injury, as has been observed in autoimmune diabetes in NOD mice (53) and in some TG models of diabetes due to β cell injury (12, 13). However, this hypothesis is not strongly supported by several pieces of experimental evidence. First, envelope-specific $CD4^+$ T cells can mediate in non-TG B6 mice complete elimination of disseminated FBL tumor, in the absence of any contribution by $CD8^+$ T cells (18–20), affirming the ability of this effector population to promote tissue destruction in vivo. Second, in models of GVHD with strains of mice differing only at class II, $CD4^+$ T cells can mediate lethal GVHD (54, 55). Finally, in mice expressing a class II transgene in pancreatic acinar cells, T cells restricted to this novel class II antigen can mediate pancreatic tissue destruction (56). Thus, $CD4^+$ T cells have demonstrable activity as effector cells in many in vivo models, presumably in part through the secretion of cytokines and activation of other cytolytic cells such as macrophages and NK cells.

Another possible explanation for the apparent lack of injury to normal tissues is that the expression of FMuLV envelope in TG lymphoid tissues is below the level required to serve as an effective target for $CD4^+$ T cell–mediated tissue damage. The FMuLV envelope protein was detected in these studies in TG lymphoid tissues at a lower level than that found in FBL tumor cells. However, TG env^+ lymphocytes were capable of stimulating in vitro proliferation by envelope-reactive immune T cells and the envelope-specific B10 clone (Fig. 4). Thus, the same TG lymphocytes that induce tolerance to priming in TG mice can stimulate primed envelope-reactive T cells in vitro, but appear to be neither stimulatory nor susceptible to injury by primed envelope-reactive T cells in vivo. Potentially, the in vitro stimulatory conditions reflect conditions disparate from those encountered in vivo with TG lymphocytes, and current studies are exploring if this reflects concentration of antigen and/or the stimulatory or processing activity of a distinct accessory cell or APC.

The results provide encouragement for pursuing TAA detected in limited amounts in normal tissues as potential targets for T cell tumor therapy. Studies in TG mouse lines expressing other proteins have demonstrated that it may be possible to induce in vitro T cell responses to antigens encoded by transgenes despite apparent nonreactivity in vivo. The use of selected APC in vitro, such as dendritic cells that can stimulate primary in vitro T cell responses (57), may improve the ability to generate such responses. Alternatively, immunization in vivo with infectious viruses containing the protein of interest (12, 13), or with concurrent administration of exogenous IL-2 to overcome an initially deficient helper T cell response (58), has been shown to induce T cell responses to presumably

tolerogenic TG proteins. In our studies, a recombinant vaccinia virus failed to elicit responses to the transgenic TAA, but other viruses need to be explored. Further studies in this and related lines of TG mice, in which the expression of TAA is controlled by promoters resulting in selective expression in nonlymphoid tissues, may help identify principles for the development of methods to generate tumor-reactive T cells with potential therapeutic activity.

We thank Sandy Emery and Kent Slaven for their expert technical help, and Joanne Factor and Anita Rogers for their assistance in preparation of this manuscript.

This study was supported by National Cancer Institute grant CA-33084 and by ZymoGenetics, Inc.

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Received for publication 21 January 1993 and in revised form 2 March 1993.

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