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.

Effectors 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; E#z, heavy chain / enhancer; FBL, Friend virus–induced leukemia of B6 origin; FMuLV, Friend murine leukemia virus; hGH, human growth hormone; P#z, heavy chain # Vh promoter; SA, splice acceptor site; SD, splice donor site; TAA, tumor-associated antigen; TG, transgenic.
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 enhancer (Eμ) and Vh promoter (Pμ) 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 envelope proteins 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-2b, Thy-1.2, denoted B6) mice and breeding pairs of congenic B6.PL (74NS) (H-2b, Thy-1.1, denoted B6/Thy-1.1) mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Cell Lines and Viruses. FMuLV env- and gag-encoded products and class I but not class II H-2b 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 plg-ENV-human growth hormone (hGH) fusion gene (Fig. 1) was constructed using a multi-step procedure. A 2.2-kb EcoRI-Sts1 fragment of plasmid PICμPR8E8 (W. Kindsvogel, ZymoGenetics, Seattle, WA), containing the combined Eμ and Pμ sequences derived from the mouse Ig heavy chain gene (23, 24), was cloned into PUC19 to generate plasmid PJH1. A 2.2-kb Xbal-Pst1 fragment of FMuLV clone 57, containing the entire FMuLV env gene (25), was subcloned into PUC18 to generate plasmid PVK4. A 2.2-kb Xbal-HindIII fragment, containing the FMuLV env gene, was then isolated from PVK4 and inserted into the XbaI and HindIII sites of BJH1, immediately downstream of the Eμ/Pμ segment, to generate plasmid BJH2. 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 BJH2. The final plasmid (plg-ENV-hGH) contains the Eμ/Pμ regulatory elements, the FMuLV env gene and hGH gene oriented 5' to 3' (Fig. 1). A 6.5-kb EcoRI fragment of plg-ENV-hGH was isolated for microinjection.

The plg-ENV-3'LTR fusion gene was constructed in multiple steps. Complementary 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' SalI site of a 4.1-kb SplH-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 Sall-HindIII sites to generate plasmid P1H1. Finally, a 2.2-kb EcoRI-Sall fragment of P1H1, which contains the combined Eμ/Pμ regulatory sequence, was inserted between the EcoRI and SplH sites of P1H1, immediately upstream of the FMuLV env gene. The final plasmid (plg-ENV-3'TLTR) contains the Eμ/Pμ control elements and the FMuLV env-3'LTR gene (Fig. 1). A 6.3-kb EcoRI-HindIII fragment of plg-ENV-3'TLTR 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 plg-ENV-3'TLTR in pups was determined by hybridization of a Southern blot of PstI-digested tail DNA to a 32P-labeled 870-bp BamHI-Pst1 fragment of the FMuLV env gene probe (25). Mice containing the plg-ENV-hGH trans-
gene were determined by dot blot analysis (30) using a 32P-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 guanidinium-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 32P 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 Hx hydrazide gel column (Bio-Rad Laboratories, Richmond, CA) (35). Spleen cells from plg-ENV TG mice or non-TG littermates were resuspended in buffer containing 20 mM Heps (pH 7.3), 1 mM EDTA, 50 mM NaCl, and 0.4% NP40. The whole cell lysate was 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 dry milk/100 mM Tris-HCl, 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 125I-labeled affinity-purified rabbit anti–goat Ig (2 × 105 cpm/ml) in TTBS.

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**Immunization.** 6–8-wk-old mice were inoculated via tail scratch with 109 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 proteins were evaluated as described (36). Briefly, in vivo primed spleen responders were cultured with either irradiated stimulator cells or UV-inactivated virus in 96-well plates by passage through nylon wool columns (34).

**Adoptive Cell Transfer.** Immune splenocytes were obtained from B6/Thy-1.1 mice immunized with vac-env or vac as described above. 106 spleen cells were injected intraperitoneally into plg-ENV TG mice and non-TG control mice. To promote in vivo proliferation of transferred immune T cells, irradiated FBL tumor cells (107) and human rIL-2 (5,000 U/d for 5 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. Veterinary pathologists reviewed the slides to evaluate the lymphoid tissues for pathologic injury.

**Analysis of Peripheral Blood T and B Cells on the FACScan.** 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., Burlingon, CA) and analyzed on the FACScan (39).

**Adaptive Chemioimmunotherapy (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, plg-ENV TG mice were inoculated intraperitoneally with 5 × 105 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 plg-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 plg-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, plg-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
microinjection of the 6.5-kb linearized plg-ENV-hGH hybrid DNA fragment into pronuclei of B6 mouse zygotes, 30 pups were born, with two containing the integrated plg-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 32P-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 plg-ENV TG mice, 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 gp85env and the processed glycosylated protein gp70env 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, BFL 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.
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 vacc-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 vacc-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 vacc-env-primed TG mice exhibited no response to either preparation of FMuLV envelope antigen. TG T cells primed with vacc-env responded normally to vaccinia virus, affirming that such T cells were infected with vaccinia virus and were competent to respond.

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 x 10⁶ viable FBL tumor cells (Table 2). Prior immunization with vacc-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, vacc-env-immunized TG mice remained susceptible to FBL tumor challenge, with all mice dying of progressive tumor growth by day 20. Moreover, vacc-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 congeneric B6/Thy-1.1 donor mice primed with vacc-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 plg-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.

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

| Donor cells* | Recipients | Additional treatment* | Histology of lymphoid tissues | Blood lymphocyte counts† |
|--------------|------------|-----------------------|-------------------------------|--------------------------|
|              |            | FBL, rIL-2            |                               | T cells | B Cells |
|              |            |                       |                               | Day 0 | Day 28 | Day 0 | Day 28 |
| 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 10⁵ 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 (10⁷) were injected intraperitoneally into indicated plg-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.

Expression of Tumor Antigens in Transgenic Mice
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 mice (six mice/group) were inoculated intraperitoneally with 5 x 10⁶ 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 x 10⁷ vac-primed B6 T cells, or CY plus 5 x 10⁷ 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-2Kb TG mice showed that expression of the H-2Kb transgene in peripheral tissue could tolerize adoptively transferred H-2Kb-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 x 10⁶ 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 x 10⁷ splenic T cells from vac-primed B6 mice had no significant effect on survival. In contrast, therapy with CY plus 5 x 10⁷ 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.

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

| Responder | Adoptive transfer | Immunization | FBL | FMuLV | Vac | Δcpm |
|-----------|-------------------|--------------|-----|-------|-----|------|
| TG        | vac-env-primed B6 spleen cells | vac-env | 6,062 | 5,203 | 52,206 | |
| TG        | Medium | vac-env | 524 | -423 | 51,841 | |

* Plg-ENV TG mice injected intraperitoneally with medium or 10⁸ 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 autoimmunity in TG mice.

The TG mice were constructed by introducing the FMuLV env gene into B6 mice under the control of the Ig Eμ/μ 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 Vβ gene usage by T cells responsive to FMuLV envelope has failed to detect a dominant Vβ 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 plg-ENV TG mice did not result in any apparent autoimmune disease, as reflected by the absence 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 vaccinia 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.

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References

1. Schreiber, H., P.L. Ward, D.A. Rowley, and H.J. Strauss. 1988. Unique tumor-specific antigens. Annu. Rev. Immunol. 6:465.
2. Hellström, K.E., and J. Hellström. 1989. Oncogene-associated tumor antigens as targets for immunotherapy. FASEB (Fed. Am. Soc. Exp. Biol.) J. 3:1715.
3. Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. Cell. 49:273.
4. Kistelow, P., H. Bluthman, U.D. Staerz, M. Steinmetz, and I. Schreiber, H., P.L. Ward, D.A. Rowley, and H.J. Strauss. 1988. Tolerance in T cell receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. Nature (Lond.). 333:742.
5. Schwartz, R.H. 1989. Acquisition of immunologic self-tolerance. Cell. 57:1073.
6. Lo, D., L.C. Burkly, G. Widera, C. Cowing, R.A. Flavell, R.D. Palmiter, and R.L. Brinster. 1988. Diabetes and tolerance in transgenic mice expressing class II MHC molecules in pancreatic beta cells. Cell. 53:159.
7. Markmann, J., D. Lo, A. Naji, R.D. Palmiter, R.L. Brinster, and E. Heber-Katz. 1988. Antigen presenting function of class II MHC expressing pancreatic beta cells. Nature (Lond.). 336:476.
8. Schönrich, G., U. Kalinke, F. Mombaugh, M. Malissen, A.-M. Schmitt-Vrhlst, B. Malissen, G.J. Hammerling, and B. Arnold. 1991. Downregulation of T cell receptors on self-reactive T cells as a novel mechanism for extrathymic tolerance induction. Cell. 65:293.
9. Morahan, G., J. Allison, and J.F.A.P. Miller. 1989. Tolerance of class I histocompatibility antigens expressed extrathyrmically. Nature (Lond.). 339:622.
10. Bohme, J., B. Schuhbaur, O. Kanagawa, C. Bencio, and D. Mathis. 1990. MHC-linked protection from diabetes dissociated from clonal deletion of T cells. Science (Wash. DC). 249:293.
11. Murphy, K.M., C.T. Weaver, M. Elish, P.M. Allen, and D.Y. Loh. 1989. Peripheral tolerance to allogeneic class II histocompatibility antigens expressed in transgenic mice: evidence against a clonal-deletion mechanism. Proc. Natl. Acad. Sci. USA. 86:10034.
12. Ohashi, P.S., S. Oehen, K. Buerki, H. Pircher, C.T. Ohashi, B. Odermatt, B. Malissen, R.M. Zinkernagel, and H. Halfartner. 1991. Ablation of “tolerance” and induction of diabetes by virus infection in viral antigen transgenic mice. Cell. 65:305.
13. Oldstone, M.B.A., M. Nerenberg, P. Southern, J. Price, and H. Lewicki. 1991. Virus infection triggers insulin-dependent diabetes mellitus in a transgenic model: role of anti-self (virus) immune response. Cell. 65:319.
14. Schild, H., O. Rötzschke, H. Kalbacher, and H.-G. Rammensee. 1990. Limit of T cell tolerance to self proteins by peptide presentation. Science (Wash. DC). 247:1587.
15. Gammon, G., and E. Sercarz. 1989. How some T cells escape tolerance induction. Nature (Lond.). 342:183.
16. P. Van Der Bruggen, C. Traversari, P. Gomez, C. Lurquin, E. De Plan, H. Van Den Eynde, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science (Wash. DC). 254:1643.
17. Greenberg, P.D. 1991. Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. Adv. Immunol. 19:281.
18. Greenberg, P.D., D.E. Kern, and M.A. Cheever. 1985. Therapy of disseminated murine leukemia with cyclophosphamide and immune Lyt-1+,2- T cells: tumor eradication does not require participation of cytotoxic T cells. J. Exp. Med. 161:1122.
19. Klarinet, J.P., D.E. Kern, K. Okuno, C. Holt, F. Lilly, and P.D. Greenberg. 1989. FBL-reactive CD8+ cytotoxic and CD4+ helper T lymphocytes recognize distinct Friend murine leukemia virus-encoded antigens. J. Exp. Med. 169:457.
20. Greenberg, P.D., M.A. Cheever, and A. Fefer. 1981. Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1-,2+ lymphocytes. J. Exp. Med. 154:952.
21. Greenberg, P.D. 1986. Therapy of murine leukemia with cyclophosphamide and immune Lyt-2+ T cells: cytolytic T cells can mediate eradication of disseminated leukemia. J. Immunol. 136:1917.
22. Earl, D.E., B. Moss, K. Wehrly, J. Nishio, and B. Chesebro. 1986. T cell priming and protection against Friend murine leukemia by a recombinant vaccinia virus expressing the env
gene. Science (Wash. DC). 234:728.

23. Gillies, S.D., S.L. Morrison, V.T. Oi, and S. Tonegawa. 1983. A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell. 33:717.

24. Grosschedl, R., and D. Baltimore. 1985. Cell-type specificity of immunoglobulin gene expression is regulated by at least three DNA sequence elements. Cell. 41:885.

25. Koch, W., G. Hunsmann, and R. Friedrich. 1983. Nucleotide sequence of the envelope gene of Friend murine leukemia virus. J. Virol. 45:1.

26. Seeburg, P.H. 1982. The human growth hormone gene family: nucleotide sequences show recent divergence and predict a new polypeptide hormone. DNA (NY). 1:239.

27. Chaffin, K.E., C.R. Beals, T.M. Wilkie, K.A. Forbush, M.I. Simon, and R.M. Perlmutter. 1990. Dissection of thymocyte signaling pathways by in vivo expression of pertussis toxin ADP-ribosyltransferase. EMBO (Eur. Mol. Biol. Organ.) J. 9:3821.

28. Beveren, C.A., J. Coffin, and S. Hughes. 1985. Nucleotide sequences complemented with functional and structural analysis In RNA Tumor Viruses. Molecular Biology of Tumor Viruses, Part 2. R. Weiss, N. Teich, H. Varmus, and J. Coffin, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 567-1148.

29. Hogan, B., F. Costantini, and E. Lacy. 1986. Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

30. Brinster, R.L., H.Y. Chen, M.E. Trumbauer, M.K. Yale, and R.D. Palmiter. 1989. Tolerance in transgenic mice expressing class II major histocompatibility complex on pancreatic acinar cells. Proc. Natl. Acad. Sci. USA. 86:3782.

31. Chirgwin, J.M., A.E. Przybyla, R.J. MacDonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid by protein-dase from sources enriched in ribonuclease. Biochemistry. 18:5294.

32. Feinberg, A.P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6.

33. Wysocki, L.J., and V.L. Sato. 1978. "Panning" for lymphocytes: a method for cell selection. Proc. Natl. Acad. Sci. USA. 75:2844.

34. Julius, M.H., E. Simpson, and L.A. Herzenberg. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol. 3:645.

35. Matson, K.S., and M.C. Little. 1988. Strategy for the immobilization of monoclonal antibodies on solid-phase supports. J. Chromatogr. 458:67.

36. Kern, D.E., D.J. Peace, J.P. Klarnet, M.A. Cheever, and P.D. Greenberg. 1988. IL-4 is an endogenous T cell growth factor during the immune response to a syngeneic retrovirus-induced tumor. J. Immunol. 141:2824.

37. Gillis, S., M.M. Ferm, W. Ou, and K.A. Smith. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. J. Immunol. 120:2027.

38. Chen, W., V.A. Reece, and M.A. Cheever. 1990. Adoptively transferred antigen-specific T cells can be grown and maintained in large numbers in vivo for extended periods of time by intermittent restimulation with specific antigen plus IL-2. J. Immunol. 144:3659.

39. Greenberg, P.D., and M.A. Cheever. 1984. Treatment of disseminated leukemia with cyclophosphamide and immune cells: tumor immunity reflects long-term persistence of tumor-specific donor T cells. J. Immunol. 133:3401.

40. Fefer, A., A.B. Einstein, M.A. Cheever, and J.R. Berenson. 1976. Models for syngeneic adoptive chemoimmunotherapy of murine leukemias. Ann. NY Acad. Sci. 276:573.

41. Shulman, M., C.D. Wilde, and G. Köhler. 1978. A better cell line for making hybridomas secreting specific antibodies. Nature (Lond.). 276:269.

42. Brinster, R.L., J.M. Allen, R.R. Behringer, R.E. Geline, and R.D. Palmiter. 1988. Introns increase transcriptional efficiency in transgenic mice. Proc. Natl. Acad. Sci. USA. 85:836.

43. Sandgren, E.P., N.C. Leutteke, R.D. Palmiter, R.L. Brinster, and D.C. Lee. 1990. Overexpression of TGF-β in transgenic mice: induction of Epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast. Cell. 61:1121.

44. Lerner, R.A., P.J. McConhey, and F.J. Dixon. 1971. Quantitative aspects of plasma membrane associated immunoglobulin in clones of diploid human lymphocytes. Science (Wash. DC). 173:60.

45. Sissons, J.G.P., M.B.A. Oldstone, and R.D. Schreiber. 1980. Antibody-independent activation of the alternative complement pathway by measles virus-infected cells. Proc. Natl. Acad. Sci. USA. 77:559.

46. Harlow, E., and D. Lane. 1988. Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 514-552.

47. Bookman, M.A., R. Swerdlow, and L.A. Matis. 1987. Adoptive chemoimmunotherapy of murine leukemia with helper T lymphocyte clones. J. Immunol. 139:3166.

48. Oliner, H., R. Schwartz, and W. Dameshek. 1961. Studies in experimental autoimmune disorders. I. Clinical and laboratory features of autoimmunization (runt disease) in the mouse. Blood. 17:20.

49. Morahan, G.E. Brennan, P.S. Bhattachal, J. Allison, K.O. Cox, and J.F.A.P. Miller. 1989. Expression in transgenic mice of class I histocompatibility antigens controlled by the metallothionein promoter. Proc. Natl. Acad. Sci. USA. 86:3782.

50. Tepper, R.I., D.A. Levinson, B.Z. Stanger, J. Campos-Torres, A.K. Abbas, and P. Leder. 1990. IL-4 induces allergic-like inflammatory disease and alters T cell development in transgenic mice. Cell. 62:457.

51. Nossal, G.J.V. 1989. Immunologic Tolerance: Collaboration between antigen and lymphokines. Science (Wash. DC). 245:147.

52. Shimizu, Y., A. van Seventer, K.J. Horgan, and S. Shaw. 1990. Roles of adhesion molecules in T-cell recognition: fundamental similarities between four integrins on resting human T cells (LFA-1, VLA-4, VLA-5, VLA-6) in expression, binding and costimulation. Immunol. Rev. 114:109.

53. Bendelac, A., C. Carnaud, C. Boitard, and J.F. Bach. 1987. Syngeneic transfer of autoimmune diabetes from NOD mice to healthy neonates. Requirement for both L3T4+ and Lyt-2+ T cells. J. Exp. Med. 166:823.

54. Korngold, R., and J. Sprent. 1985. Surface markers of T cells cause lethal graft-vs-host disease to class I vs class II H-2 differences. J. Immunol. 135:3004.

55. Thiele, D.L., S.E. Bryde, and P.E. Lipsky. 1988. Lethal graft-vs-host disease induced by a class II MHC antigen only disparity is not mediated by cytotoxic T cells. J. Immunol. 141:3377.

56. Lo, D., L.C. Burkly, R.A. Flavell, R.D. Palmiter, and R.L. Brinster. 1989. Tolerance in transgenic mice expressing class II major histocompatibility complex on pancreatic acinar cells. J. Exp. Med. 170:87.

57. Steinman, R.M. 1991. The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. 9:271.

58. Dallmann, M.J., O. Shiko, T.H. Page, K.J. Wood, and P.J. Morris. 1991. Peripheral tolerance to alloantigen results from altered regulation of the interleukin 2 pathway. J. Exp. Med. 173:79.