ABSTRACT

Immunotherapy of melanoma by adoptive transfer of tumor-reactive T lymphocytes aims at increasing the number of activated effectors at the tumor site that can mediate tumor regression. The limited span of human T lymphocytes, however, hampers obtaining sufficient cells for adoptive transfer therapy. We have shown previously that the life span of human T cells can be greatly extended by transduction with the human telomerase reverse transcriptase (hTERT) gene, without altering antigen specificity or effector function. We developed a murine model to evaluate the efficacy of hTERT-transduced human CTLs with antitumor reactivity to eradicate autologous tumor cells in vivo. We transplanted the human melanoma cell line melAKR or melAKR-Flu, transduced with a retrovirus encoding the influenza virus/HLA-A2 epitope, in RAG-2−/− Il-2Rγ−/− double knockout mice. Adoptive transfer of the hTERT-transduced influenza virus-specific CTL clone INFA24 or clone INFA13 inhibited the growth of melAKR-Flu tumors in vivo and not of the parental melAKR melanoma cells. Furthermore, the hTERT-transduced clone INFA13 inhibited tumor growth to the same extent in vivo as the untransduced clone INFA24, as determined by in vivo imaging of luciferase gene-transduced melAKR-Flu tumors, indicating that hTERT did not affect the in vivo function of CTL. These results demonstrate that hTERT-transduced human CTLs are capable of mediating antitumor activity in vivo in an antigen-specific manner. hTERT-transduced MART-1-specific CTL clones AKRd98 and AKR103 inhibited the growth of syngeneic melAKR tumors in vivo. Strikingly, melAKR-Flu cells were equally killed by the MART-1-specific CTL clones and influenza virus-specific CTL clones in vitro, but only influenza-specific CTLs were able to mediate tumor regression in vivo. The influenza-specific CTL clones were found to produce higher levels of IFNγ on tumor cell recognition than the MART-1-specific CTL clones, which may result from the higher functional avidity of the influenza virus-specific CTL clones. Also, melAKR-Flu tumors were growing faster than melAKR tumors, which may have surpassed the relatively modest antitumor effect of the MART-1-specific CTL, as compared with the influenza virus-specific CTL. Taken together, the adoptive transfer model described here shows that hTERT-transduced T cells are functional in vivo, and allows us to evaluate the balance between functional activity of the CTL and tumor growth rate in vivo, which determines the efficacy of CTLs to eradicate tumors in adoptive transfer therapy.

INTRODUCTION

The goal of immunotherapy is to bolster the immune system of the patient in such a way that it eradicates an established tumor. One way to achieve this goal is to increase the number of activated effector cells at the tumor site by adoptive transfer of in vitro generated and expanded CTLs. Indeed, infusion of human T cells has been shown to cause a delayed tumor growth in human xenograft mouse models (1–7). More importantly, adoptive transfer therapy has been successful in clinical settings as well. Transfer of virus-specific T cells has been shown to be effective in preventing reactivation of latent cytomegalovirus infections in patients after organ transplantation (8, 9) and in the treatment of lymphoproliferative disorders caused by EBV infection (10, 11). Adoptive transfer therapy of cancer requires the isolation of T cells with tumor reactivity. Tumor-infiltrating lymphocytes may be enriched for such T cells and were shown to be effective in mediating tumor regression in metastatic renal cell carcinoma patients after nephrectomy (12). Recently, the infusion of polyclonal T lymphocytes that were expanded from the tumor-infiltrating lymphocytes, combined with high doses of IL-2, has been shown to induce substantial tumor regression in metastatic melanoma patients (13). In addition, Yee et al. (14) reported regression of individual tumor metastases by adoptive transfer of CD8+ T-cell clones that recognize the melanoma antigens MART-1 or gp100 in combination with low doses of IL-2 in metastatic melanoma patients. These clinical studies show that adoptive T-cell therapy is feasible to treat cancer patients, has low toxicity, and can be effective in mediating tumor regression.

Adoptive transfer of tumor-specific CTLs has required large doses of at least 10⁸–10¹⁰ T cells. This means that, after isolation from peripheral blood or tumor-infiltrating lymphocytes, CTLs need to be expanded in vitro. Whereas it is in general not a problem to expand freshly isolated polyclonal T cells to very large numbers, this is not the case with well-defined tumor antigen-specific T-cell clones. The application of adoptive transfer therapy of well-defined antigen-specific T-cell clones is, therefore, limited by the relatively low success rate of isolating sufficient numbers of specific T cells from individual patients. CTLs with tumor reactivity that are found in cancer patients are derived frequently from the memory T-cell pool. Human CD8+CD28− memory T cells have a replicative life span of maximally 40 population doublings (PDs) in vitro but most often much less (15), which limits large-scale expansion of these cells. For initial screening approximately 10⁵-10⁶ cells are needed, which amounts to 17–20 PD when starting from one cell. Therefore, the isolation and cloning of tumor-reactive T cells selects for the relatively young T cells, or rare T cells with an exceptionally long life span, which may not be found in all cancer patients. Moreover, the limited life span of human T cells may also have contributed to the fact that the most prominent antitumor responses seen to date were obtained with relatively young cultures of tumor-infiltrating lymphocytes (16).

We have described previously that ectopic expression of the enzyme complex, telomerase reverse transcriptase (hTERT) greatly extends the life span of both human CD8+ and CD4+ T cells (17–19). Ectopic hTERT expression prevents telomere shortening in the cells, which occurs at each cell division or by oxidative DNA damage. Telomeres are DNA repeats at the distal ends of the chromosomes, which protect against chromosome end-to-end fusions (20). Critically short telomeres have an impaired function and may lead to cell cycle...
arrest. Since murine T cells have longer telomeres, resulting in a longer life span than human T cells, T-cell life span generally does not limit adoptive transfer experiments of murine T cells. Interestingly, human T cells express hTERT upon activation, allowing repair of short telomeres during activation and proliferation (21). We have shown previously that during prolonged proliferation in vitro, T cells lose the ability to up-regulate hTERT expression, and the level of telomerase activity becomes insufficient to repair the telomere erosion (19). Moreover, we observed lower levels of hTERT expression in activated memory cells, as compared with activated naive cells of the same donor (19). This indicates that the loss of hTERT expression also occurs upon proliferation in vivo, which reduces the proliferative capacity of memory T cells, as compared with naive T cells. Because tumor-reactive T cells may be more frequently found in the memory T-cell pool, transduction of memory T cells with hTERT provides a tool to overcome the limitation of a reduced proliferative capacity. We have observed that hTERT-transduced T cells retain their antigen specificity and effector function upon activation in vitro (17, 18).

Furthermore, we observed that proliferation of hTERT-immortalized T cells in vitro remained dependent on activating signals and cytokines, which underlines the notion that ectopic hTERT expression allows the continuation of proliferation (17, 18), but does not promote entry into cell cycle by itself, nor does it cause growth deregulation (22). Ectopic hTERT expression in combination with stimulation of T cells therefore enables large-scale cultures and serial cloning to isolate human T cells of desired specificity in sufficient numbers for adoptive transfer (17, 18, 23, 24). Moreover, ectopic hTERT expression allows large-scale expansion of those tumor-specific T-cell clones, which would otherwise not expand to sufficient numbers due to telomere erosion. Thus, hTERT transduction will enlarge the repertoire of CTLs that can be used for adoptive transfer. Having solved the problem of the low success rate in obtaining high numbers of cloned CTLs, it was important to show that these hTERT-transduced T cells were effective in vivo. We developed an in vivo model to test the efficacy of human hTERT-transduced CTL clones to eradicate autologous melanoma cells in an in vivo environment. In the present report, we describe adoptive transfer of hTERT-transduced CTL clones in RAG-2−/− IL-2Rγ−/− (RAGγKO) mice bearing human melanoma lung tumors. The effect of single or multiple doses of two influenza-reactive CTL clones or two MART-1-reactive CTL clones on the growth of a human melanoma in vivo was studied in relation to their functional activity in vitro. In this model, the criteria for CTL clones to be effective in mediating tumor regression in vivo upon adoptive transfer can be defined.

**MATERIALS AND METHODS**

**Mice.** Male RAG-2−/− IL-2Rγ (common γ-chain)−/− double knockout (RAGγKO) mice on a C57/B16 background, as described previously (25), were used at the age of 6–8 weeks. These mice have no functional T, B, and natural killer cells, and are not leaky for these cell types. These mice can be maintained as double knockout mice. The mice were bred under specific pathogen-free conditions, maintained in isolators, and all of the manipulations were performed under laminar airflow.

**Tumor Cell Lines.** The melanoma cell line melAKR was derived from a melanoma lesion of patient AVL-3 (26). MeiAKR-Flu was derived by transduction of melAKR with the retrovirus encoding the influenza matrix peptide GILGFVFTL that binds to HLA-A2 molecules, a series of murine CTL epitopes, and the green fluorescent protein (GFP) gene connected by the internal ribosomal entry site (IRES) sequence, as described (27). MeiAKR, melAKR-Flu, the EBV-transformed B cell line JY, which expresses HLA-A2, and the erythroleukemia cell line K562 were cultured in Iscove’s modified Dulbecco’s medium (Invitrogen Life Technologies, Breda, The Netherlands), supplemented with 8% FCS (Invitrogen Life Technologies), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Roche Diagnostics, Mannheim, Germany).

**Isolation of Human T-Cell Clones.** CD8+ T-cell clones INF13 and INF14 were derived from the peripheral blood mononuclear cell (PBMC) of an HLA-A2-positive healthy donor. CD8+ T cells isolated from the PBMCs by MACS sort using anti-CD8 antibody (Ab)-coated beads, were stimulated with the CD8-negative PBMC fraction pulsed with the influenza virus matrix peptide 58–66 (GILGFVFTL) in the presence of 20 IU/ml recombinant human rIL-2 (Proleukin, Chiron, Amsterdam, the Netherlands). Subsequently, CD8+ T cells that recognize the influenza peptide in HLA-A2 were detected by binding of the HLA-A2/influenza tetramer, and cloned by fluorescence activated single cell sorting (FACSstar Plus; Becton Dickinson, San Jose, CA). Clones INF13A and INF14 were identified to specifically recognize influenza virus matrix peptide 58–66 (GILGFVFTL) on HLA-A2-positive target cells. The T-cell receptor (TCR) Vβ chain expressed by the T-cell clones was determined by TCR Vβ chain-specific Ab staining (I0est β Mark; TCR Vβ Repertoire kit; Immunotech, Marseille, France). T-cell clones INF13A AND INF14 were both characterized by the expression of TCR Vβ17, which has been shown previously to be used by human influenza-specific T-cell clones (28).

CD8+ T-cell clone AKR4D8 is a subclone of clone AKR4 that was derived from patient AVL-3 (26) after stimulating PBMCs with the autologous melanoma cell line melAKR that was genetically engineered to produce IL-7 (17). After stimulation, the cells were cloned by single cell sorting, and clone AKR4 was identified to recognize the MART-1 peptide analog 26–35 (ELAGIGILTV) that binds to HLA-A2 molecules (29), and to a lesser extent the unmodified MART-1 epitope 26–35 (EAAGIGILTV). Clone AKR4D8 was isolated from two consecutive rounds of subcloning of clone AKR4. As expected, the AKR4D8 subclone was also reactive with the MART-1 epitope presented in HLA-A2 (17). CD8+ T-cell clone AKR103 was derived from the PBMC of patient AVL-3 that had been stimulated with the autologous melanoma line melAKR transduced with the costimulatory molecule CD80, and subsequently cloned by single cell sorting.1 Clone AKR103 was identified to recognize both the MART-1 peptide 26–35 EAAAGILTV, as well as the MART-1 peptide analog ELAGIGILTV, presented by HLA-A2. T-cell clone AKR4D8 expressed TCR Vβ8, whereas clone AKR103 did not bind to any of the Vβ chain-specific antibodies of the TCR Vβ repertoire kit (I0est β Mark; TCR Vβ Repertoire kit; Immunotech), indicating that the clones AKR4D8 and AKR103 represented different CTL clones isolated from patient AVL-3.

**T-Cell Culture and Transduction.** T-cell clones were cultured in Yssel medium (30), supplemented with 1% human serum, 20 IU/ml rIL-2, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded weekly at 0.3 × 10^6 cells/ml in the presence of a feeder mixture consisting of 0.1 × 10^5/ml irradiated (80 Gy) JY cells, 1 × 10^6/ml irradiated (40 Gy) allogeneic PBMC, and 0.1 × 10^6 cells/ml irradiated (80 Gy) meliAKR-Flu cells. Alternatively, the meliAKR-Flu cells in the feeder mixture were replaced by 100 ng/ml phytohemagglutinin (HA16; Murex Biotech, Dartford, United Kingdom) in one stimulation every 3 weeks. Cultures were performed in 24-well plates at 1 ml/well, or in 125-cm² tissue culture flasks containing 150–300 ml culture volume (1–2 ml culture volume/cm²). All of the CTL clones were transduced with a retrovirus encoding the hTERT gene and the GFP gene connected by the IRES sequence, as described (17). Briefly, T cells were stimulated with the feeder cell mixture containing phytohemagglutinin, as described above, 2 days before transduction. The cells were transduced with supernatant containing the retrovirus encoding hTERT-IRES-GFP, in fibronectin fragments-coated plates (Retronectin, Takara, Japan) in the presence of 20 IU/ml rIL-2. During transduction the plate was spun at 2500 rpm for 90 min at 25°C. Subsequently, half of the transduction supernatant was replaced by freshly thawed retroviral supernatant, and the transduction was cultured overnight at 37°C and 5% CO₂. After transduction, the cells were washed and cultured, as described above. hTERT expression by the transduced CTL clones was determined as the GFP expression level by flow cytometry.

1 E. Hoogenberg and J. J. Ruizerenda, unpublished observations.
Preincubation of target cells with 100 μM peptide was performed during chromatin labeling followed by three wash steps. For functional avidity testing, the peptides were added to the test in 10-fold dilutions ranging from 10^2 to 10^5. All assays were performed in a volume of 150 μl/test. 3Ci release was measured in 25 μl of the test supernatant dried on a LUMA scintillation plate (Bio-Rad, Hercules, CA) in a β-radiation counter (Topcount NXT; Packard, Randburg, South Africa).

**HLA-Peptide Tetramers.** Allophycocyanin-conjugated tetramers composed of HLA-A2 and the MART-1 peptide analog 26–35 (ELAGIGILTV) or the influenza virus matrix peptide 58–66 (GILGFVFTL) were synthesized as described (32). Binding of tetramers to T cells was tested by incubation of 2 × 10^5 T cells with 0.1 μg tetramer for 10 min at 37°C, followed by incubation with phycoerythrin-conjugated anti-CD4, anti-CD8, or anti-TCRβ Abs (Becton Dickinson) on ice for 30 min. Cells were analyzed by flow cytometry on a FACSCalibur (Becton Dickinson). Propidium iodide was added to exclude nonviable cells.

**ELISA.** T cells (3300 cells/well) and tumor cells (6600 cells/well) were cocultured overnight in triplicate cultures in a 96-well round-bottomed plate in a total volume of 200 μl/well Yssel's medium, supplemented with 1% human serum, 20 IU/ml rhIL-2, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells were preincubated with 100 μM influenza virus matrix peptide 58–66 (GILGFVFTL) for clones INFA13 and INFA24, or with the MART-1 peptide 26–35 (ELAGIGILTV) for clones AKR4D8 and AKR103, washed, and cocultured with T cells. The supernatants were collected after overnight culture and analyzed for the presence of IL-4, IL-10, and IFNγ by ELISA. The concentration of IL-4, IL-10, or IFNγ in the supernatant was determined in triplicate by cytokine-specific ELISA (PeliKine; Sanquin Reagents, Amsterdam, the Netherlands) using a standard curve of diluted recombinant cytokine provided in the kit.

**Adoptive Transfer Protocol.** Six to 8-week-old male RAG/γκKO mice were injected i.v. in the tail vein with 1 × 10^6 or 0.5 × 10^6 melAKR-Flu-tumor cells. The mice were treated with a dose of 5 × 10^6 T cells by i.v. injection in the tail vein on day 3, followed by a s.c. rhIL-2 depot in the flank. This rhIL-2 depot consists of a suspension of 2 × 10^4 IU rhIL-2 in 40 μl Iscove’s modified Dulbecco’s medium (Invitrogen Life Technologies), and 80 μl incomplete Freund’s adjuvant (Difco Laboratories, Detroit, IL). One group of 8 mice/experiment received another i.v. dose of 5 × 10^6 T cells in the tail vein at day 5 and at day 10. A control group of 8 mice in each experiment received only tumor cells and the rhIL-2 depot. The mice were sacrificed on day 17, and the lungs were excised. Half of the left lung was isolated and kept in medium for the detection of the injected T cells by fluorescence-activated cell sorter analysis. The rest of the lung was filled with paraformaldehyde via the trachea to open the alveoli. This manipulation increases the morphology of the lung structure and was used for immunohistochemical analysis to determine the tumor size in the lung.

**Detection of Human T Cells by Flow Cytometry.** The lung tissue was cut into small pieces and mashed into a single cell suspension, followed by total lymphocyte isolation on a Ficoll gradient. After washing, the cells were incubated with phycoerythrin-conjugated antimurine CD45 Ab (Becton Dickinson), PerCP-conjugated antihuman CD8 Ab (Becton Dickinson), and allophycocyanin-conjugated antihuman CD45 Ab (Becton Dickinson) for 30 min on ice. Human T cells were detected as the population-expressing GFP and human CD8 expression. The kinetics of the injected human T cells was measured in groups of 8 RAG/γκKO mice injected with 0.5 × 10^6 melAKR-Flu tumor cells and treated after 3 days with a single dose of 5 × 10^6 INFA24 T cells. Two mice were sacrificed 3 h, 3 days, 7 days, or 14 days after T-cell transfer, to analyze the presence of injected T cells in the lungs and in the peripheral blood by flow cytometry. Long-term experiments of T-cell survival were performed in 14 mice injected with a single dose of 5 × 10^6 INFA24 T cells, together with a rhIL-2 depot, and sacrificed after 3 weeks, or after 3, 6, 9, or 11 months. Autopsy was performed on the mice to detect any malignancies or other abnormalities. The presence of human T cells in the lungs was analyzed by flow cytometry.

**Detection of Melanoma Cells by Immunohistochemistry.** The right lung was fixed with formalin and embedded in paraffin to cut longitudinal sections. The sections were stained with polyclonal rabbit Ab S-100 (DAKO, Glostrup, Denmark) to detect melanoma cells. Briefly, the sections were pretreated with Pronase (Sigma Aldrich, Zwijndrecht, the Netherlands) for 10 min, washed, and preincubated with 5% normal goat serum (Sanquin Reagents). Sections were subsequently incubated with the S-100 antibody overnight at 4°C in a humidified chamber. After washing, the biotinylated goat-anti-rabbit IgG Ab (DAKO) was added for 30 min. The sections were washed and incubated with a streptavidin-biotin complex conjugated to horseradish peroxidase (DAKO) for 30 min. Bound antibody was detected by incubation with 3,3′-diaminobenzidine (DAKO) for 5 min, which is visible as a brown staining pattern. The sections were counterstained with hematoxylin.

**Quantification of the Number of Microtumors and Total Tumor Size.** For each mouse, the number of microtumors was counted in two whole longitudinal lung sections of the right-side lung by two independent observers in two separate sessions. The total tumor size in the lung was quantified as the total area of S-100 staining cells in the two longitudinal lung sections per mouse, using the computer-aided detection program KS-400 (Zeiss, Weesp, the Netherlands), which measures the area of staining per vision field, which is observer independent and gives an objective analysis of the tumor size. The total tumor size was expressed as the sum of the staining area of all of the vision fields in two whole longitudinal sections that were sampled from the center of the lung tissue.

**Statistical Analysis.** Student’s t test was used to determine the significance of the differences in the number of microtumors and the total tumor size between the different groups of mice.

**In Vivo Imaging of Tumor Xenografts.** The luciferase gene isolated from the PGL3 vector (Promega, Madison, WI) was cloned into the retroviral vector pMX in an IRES-YFP configuration. MelAKR-Flu-tumor cells were transduced with a retrovirus encoding the luciferase-IRES-YFP construct. Luciferase-transduced cells (melAKR-Flu-Luc) were selected based on YFP expression by fluorescence-activated cell sorting 48 h after transduction. Adoptive transfer was performed as described above. Mice were injected with 0.5 × 10^6 melAKR-Flu-Luc cells and received an i.v. injection on day 3 with 5 × 10^6 untransduced cells or hTERT-transduced cells of CTL clone INFA13, as well as a s.c. rhIL-2 depot. Control mice received only tumor cells and the rhIL-2 depot. Tumor growth was monitored in vivo by bioluminescence imaging between day 3 and day 20. Mice were anesthetized with isoflurane (Abbott Laboratories, Queensborough, United Kingdom). An aqueous solution of the substrate luciferin (150 mg/kg; Xenogen, Alameda, CA) was injected into the peritoneal cavity 6 min before imaging. Animals were placed into the light-tight chamber of the CCD camera (IVIS; Xenogen). A gray-scale photographic image of the animal was taken in the chamber under dim illumination. After switching off the light source, the photon counts produced by active luciferase within the melAKR-Flu-Luc cells were acquired during a defined period of time ranging up to 2 min. Signal intensity was quantified as the sum of all of the detected photon counts within the region of interest after subtraction of background luminescence, using the software program Living Image (Xenogen). A pseudocolor image representing the spatial distribution of photon counts within the animal (blue, least intense and red, most intense) was generated in Living Image and overlaid on the gray-scale reference image, allowing anatomical localization of the tumors. At day 20, the mice were sacrificed and the lungs were injected via the trachea with a suspension of India ink (15% India ink and 0.01% concentrated ammonium hydroxide in distilled water). Lungs were then removed and bleached with Fekete’s solution (58% ethanol 95%, 20% distilled water, 8% formaldehyde solution 37%, and 4% glacial acetic acid). Tumor nodules appeared as discrete white nodules against the black background of normal lung tissue.

**RESULTS**

**Characterization of the Functional Activity of Human CTL Clones with Extended Life Span in Vivo.** To compare different human CTL clones for their individual efficacy to eradicate autologous human melanoma cells in vivo, we generated two influenza virus-specific CTL clones, INFA13 and INFA24, and two melanoma-specific CTL clones, AKR4D8 and AKR103. All of the CTL clones were transduced with the hTERT-IRE-5'F encoding retrovirus. During subsequent culture, we observed an accumulation of hTERT-IRE-5'F-expressing cells in all of the transduced T-cell cultures, which contained only transduced T cells after 1 or 2 months. The mean fluorescence intensities of GFP expression level of the
hTERT-IRE-GFP transduced clones AKR4D8, AKR103, INFA24, and INFA13 were 120, 310, 405, and 255, respectively. hTERT transduction extended the life span of the CTL cultures far beyond the life span of the untransduced cell cultures, which was between 38 and 42 PD for all of the CTL clones, as we have described previously (17, 18). All of the clones were 100% GFP positive, indicating they were all hTERT positive when the growth curves were determined. The CTL clones AKR4D8, AKR103, and INFA13 were growing at a comparable rate of 1.9, 1.5, and 1.8 PD per stimulation, respectively. Clone AKR103 was growing faster at a rate of 2.8 PD per stimulation. All of the hTERT-transduced clones were able to grow in large culture volumes of 150–300 ml containing 1–2 × 10^6 T cells/ml upon weekly stimulation with the feeder cell mixture to obtain sufficient T cells for adoptive transfer therapy.

CTL clones INFA24 and INFA13 lysed JY cells only when pulsed with the HLA-A2-binding influenza virus matrix peptide (58–66) GILGFVFTL (Fig. 1A). These CTL clones did not recognize HLA-A2-positive melAKR melanoma cells, but lysed melAKR-Flu cells, which express the influenza virus (58–66) epitope (Fig. 1A). HLA-A2-positive targets loaded with irrelevant peptide were not recognized by these CTL clones (data not shown). These results show that the clones specifically recognized the influenza epitope. Moreover, both CTL clones expressed CD8 and bound HLA-peptide tetramers composed of HLA-A2 and the influenza virus matrix peptide, but not HLA-A2 tetramers containing the MART-1 peptide analog (Fig. 1B). Both CTL clones INFA13 and INFA24 displayed an equally high functional avidity, as determined by the half maximal lysis of peptide-loaded JY cells at 100 pM of influenza virus matrix peptide (58–66) GILGFVFTL (Fig. 1C).

CTL clones, AKR4D8 and AKR103, both specifically lysed the autologous melanoma cell line melAKR, as well as melAKR-Flu cells (Fig. 1A). Autologous EBV-transformed B cells (data not shown) or JY cells were lysed only when preloaded with MART-1 peptide (26–35) EAAGIGILTV, or with the MART-1 peptide analog ELA-GIGILTV (Fig. 1, A and C). HLA-A2-positive melanoma cells lacking MART-1 expression were not recognized, nor JY cells loaded with irrelevant peptide (data not shown). The slightly enhanced cytolyis by clone AKR103, as compared with clone AKR4D8, may reflect a difference in the intrinsic cytolytic capacity. However, redirected cytotoxicity assays of both clones against the Fc-receptor-expressing P815 cells preloaded anti-CD3 antibody revealed that the

Fig. 1. Specific target cell recognition and tetramer binding by human telomerase reverse transcriptase-transduced MART-1 or influenza virus-specific CTL clones. A, recognition of melAKR (top), melAKR-Flu (middle), or JY (bottom) by MART-1-specific CTL clones AKR4D8 and AKR103 or influenza virus-specific CTL clones INFA24 and INFA13, was tested in a 4-h ^{51}Cr release assay. Nonspecific target cell lysis by the CTL was suppressed by a 50-fold excess of unlabeled K562 cells. ○ indicate the lysis of target cells in the absence of peptide; □ indicate the target cells lysis after preloading with MART-1 peptide analog ELA-GIGILTV in assays of clones AKR4D8 and AKR103 or influenza virus matrix peptide 58–66 (GILGFVFTL) in assays of clones INFA24 and INFA13. Results are representative of five independent ^{51}Cr release assays performed 6–8 days after stimulation of the T-cell culture. B, specific tetramer binding by the human telomerase reverse transcriptase-transduced CTL clones. Graphs show the binding of HLA-A2/MART-1 26–35 (top) or HLA-A2/influenza 58–66 (bottom) tetramers, and the expression of CD8 by CTL clones AKR4D8, AKR103, INFA24, and INFA13, as tested by flow cytometry. Graphs are representative of eight independent tetramer-binding assays performed 8–10 days after stimulation of the T-cell culture. C, functional avidity of the CTL clones. Right graph, lysis of JY cells by CTL clone AKR4D8 (squares) or clone AKR103 (circles) in the presence MART-1 peptide EAAGIGILTV (closed symbols) or peptide analog ELA-GIGILTV (open symbols) at concentrations ranging from 100 μM to 0.1 μM was tested in a chromium release assay. Graph shows the percentage specific lysis upon 4-h incubation at an E:T ratio of 30:1. Functional avidity was determined by the peptide concentration at which half the maximal lysis was observed. Clone AKR103: 10–100 nM EAAGIGILTV, 100 nM -1 μM EAAGIGILTV. Clone AKR4D8: 1–10 μM EAAGIGILTV, 100 nM EAAGIGILTV. Left graph, lysis of JY cells by CTL clone INFA13 (○) or clone INFA24 (●) in the presence influenza virus matrix peptide 58–66 (GILGFVFTL) at concentrations ranging from 10 μM to 0.1 μM at an E:T ratio of 5:1. Graphs show an equal avidity of clone INFA13 and INFA24 at 100 μM.
cytolytic capacities of these two CTL clones upon anti-CD3 cross-linking were comparable (data not shown). Although both CTL clones recognized the MART-1 peptide analog ELAGIGILTV in cytotoxicity assays, only clone AKR4D8 bound tetramers composed of the MART-1 peptide analog ELAGIGILTV and HLA-A2 molecules (Fig. 1B). Both CTL clones expressed comparable levels of TCR and CD8, as judged by anti-TCRaB or anti-CD8 antibody binding, indicating that the absence of tetramer binding of clone AKR103 was not due to a lower T-cell receptor or CD8 expression level (Fig. 1B). Additional analysis revealed that the absence of tetramer binding to clone AKR103 was most likely due to the very low affinity of its TCR for the MART-1 peptide analog ELAGIGILTV that was used to make the tetramer (Fig. 1C). Whereas clone AKR4D8 showed a high functional avidity for the MART-1 peptide analog ELAGIGILTV with a half-maximal lysis at 1–10 pm peptide (Fig. 1C), the avidity of clone AKR103 for this peptide was 10,000-fold lower (10–100 nM). A smaller difference in avidity between the CTL clones was found for the naturally processed epitope of the MART-1 protein, EAAGIGILTV, which is presented by the melAKR cells (Fig. 1C). Thus, it can be expected that both clones would bind tetramers of the natural peptide equally well. However, this could not be tested because due to the lower affinity of this natural MART-1 peptide for HLA-A2, it could not be used to generate tetramers. The difference in affinities of the AKR4D8 and the AKR103 TCR for the MART-1 peptide analog suggested already that these clones expressed different TCR. This was confirmed by specific antibody staining of the TCR Vβ chain (data not shown). The functional in vitro cytolytic activities of all four of the hTERT-transduced CTL clones was identical to that of the untransduced CTL clones, as we have described previously (17), indicating that hTERT transduction had not changed the specific target cell recognition of the CTL clones.

The cytotoxicity assays showed that melAKR-Flu cells were lysed to a similar extent by the influenza virus-specific CTL clones, and the CTL clones AKR103 and AKR4D8 (Fig. 1A). Stimulation with plate-bound anti-CD3 antibody induced comparable levels of IFNγ in all four of the CTL clones (Fig. 2), indicating that the intrinsic capacities of the influenza virus-specific and the MART-1-specific CTL clones to produce IFNγ were the same. However, the influenza virus-specific CTL clones produced 3–4-fold higher levels of IFNγ upon recognition of melAKR-Flu cells than the MART-1-specific CTL clones (Fig. 2). Likewise, recognition of JY cells loaded with specific peptide resulted in higher levels of IFNγ production by the influenza-specific clones, as compared with the MART-1-specific CTL clones. None of the CTL clones produced IL-4 or IL-10 on activation (data not shown). Although we cannot rule out that the influenza epitope expression on the melAKR-Flu cells may have been higher than the MART-1 expression, the enhanced IFNγ production by the influenza virus-specific CTL clones upon recognition of peptide loaded JY cells (Fig. 2) may have resulted from the higher avidity of these CTL clones, as compared with the MART-1 CTL clones (Fig. 1C). It is likely that the difference in avidity between the clones is caused by the higher TCR affinity of the influenza virus-specific clones and not by differences in the capacity to form adhesions, because MelAKR-Flu cells expressed CD58, CD54 (ICAM1), CD102 (ICAM2), and CD50 (ICAM3), the ligands of which, CD2, CD11a (LFA-1), activated CD11a, or CD18, were expressed at comparable levels on all four of the CTL clones.

Effect of Adoptive Transfer of Specific T Cells on the Growth of Human Melanoma in RAG/γcKO Mice. We developed a transplantation protocol of human melanoma cells in the RAG/γcKO mice. When injected i.v. in the tail vein, melAKR melanoma cells were found to grow as micrometastases in the lungs after 17 days (Fig. 3A). The micrometastases were growing in between the alveoli, extravasating from the blood vessels (Fig. 3B), as well as in the pleural cavity. The number of lung micrometastases varied between 200 and 900 in two entire longitudinal lung sections in different experiments. Injection of 1 × 10^6 melAKR cells gave rise to tumors in all of the mice, which were efficient in number and size after 17 days to detect possible effects of treatment. The transduced cell line melAKR-Flu was observed to grow with an approximately two times shorter doubling time in culture. Injection of various tumor cell doses revealed that injection of 5 × 10^6 melAKR-Flu cells per mouse gave rise to lung microtumors at day 17 that were equivalent in number to those obtained with 1 × 10^6 melAKR cells. Therefore, experiments involving melAKR-Flu cells were performed with 0.5 × 10^6 cells/mouse.

We investigated the feasibility of this model to test the efficacy of hTERT-transduced influenza virus-specific CTL to affect the growth of tumor cells with (melAKR-Flu) or without (melAKR) specific antigen expression in vivo. Adoptive transfer of human CTL was performed 3–10 days after tumor transplantation. When a single dose of 5 × 10^6 INFA24 T cells was given i.v. at day 3, a reduction of 24% (Fig. 4A) in the number of melAKR-Flu lung microtumors and 48% decrease in total tumor size (Fig. 4A) was observed, as compared with the untreated control group. Fig. 3C illustrates the observed decrease in tumor size of the microtumors in a section of the lung after CTL treatment. The antitumor effect of the influenza-specific T cells was even more pronounced in the adoptive transfer of CTL clone INFA13, which mediated regression of 95% of the tumors (Fig. 4A). Interestingly, this difference in in vivo efficacy between the CTL clones was not apparent from the in vitro cytotoxicity assays, in which melAKR-Flu cells were lysed to the same extent by both CTL clones. As expected, treatment of melAKR tumor-bearing mice with single or repeated doses of influenza virus-specific CTL clone INFA24 neither reduced the number of micrometastases nor the total tumor size (Fig. 4A), indicating that the antitumor effect of the CTL clones was dependent on specific tumor cell recognition by the CTL. These experiments clearly demonstrated the feasibility of our in vivo model to test the efficacy of in vitro-generated and expanded CTL clones. Importantly, the experiments also show that transduction of the telomerase gene and the resulting life span extension do not lead to an abrogation of the in vivo activities of the CTL clones.
Having established that CTL clones can specifically affect tumor growth in vivo, we tested the effects of the MART-1-specific clones on the growth of wild-type melAKR tumors. Treatment of melAKR tumor-bearing mice with a single dose of clone AKR4D8 reduced the growth of melAKR microtumors. A significant reduction of 27% in the number of microtumors and 46% reduction in total tumor size were observed when three doses of CTL AKR4D8 were given (Fig. 4B). Repeated adoptive transfer of AKR4D8 T cells may, thus, reduce both tumor cell seeding and tumor growth. Injection of clone AKR103 gave a significant reduction in the number of microtumors of 24% at a single dose and of 41% at three doses (Fig. 4B), which was also evident from the significant reduction in the total tumor size, showing a reduction of 29% at a single dose and 42% at multiple doses. Tumor growth inhibition mediated by clone AKR103 was significant at a single dose of CTL, which indicates that clone AKR103 was more effective in mediating tumor regression in vivo than clone AKR4D8. Therefore, we performed additional experiments with clone AKR103.

Surprisingly, treatment of mice bearing melAKR-Flu lung tumors with one or three doses of CTL clone AKR103 did not result in any tumor regression, neither in the number of lung microtumors, nor in the total tumor size (Fig. 4B). Immunohistochemical analysis of the melAKR-Flu tumor-bearing lung sections after treatment with CTL AKR103 showed an intact expression of MART-1 in the tumor cells, indicating that the lack of tumor growth inhibition was not due to the selective loss of MART-1 antigen expression. Thus, although melAKR and melAKR-Flu cells were both lysed by clone AKR103 in vitro, clone AKR 103 failed to affect the growth of melAKR-Flu tumors in vivo. Moreover, the equal levels of melAKR-Flu and melAKR target cell lysis in vitro, render it unlikely that competition of the influenza virus peptide and the endogenous MART-1 peptide for binding to HLA-A2 decreased the recognition of melAKR-Flu cells by the MART-1-specific CTL clones in vivo. These results show that cytolytic activity of CTL in vitro is not always predictive of a
capacity to effect tumor regression in vivo. The efficacy of treatment with influenza-specific T cells indicated that melAKR-Flu melanoma tumors were susceptible to CTL treatment in vivo. It should, however, be noted that the effect of CTL on the tumor growth in vivo measured at day 17 is the net result of the inhibitory effect of the CTL and the growth rate of the tumor. The inhibitory effect of CTL AKR103 on the melAKR-Flu tumors may have been comparable with the parental melAKR tumors, similar to what was observed in cytotoxicity assays. However, as melAKR-Flu tumors were growing faster in vivo than the parental melAKR tumors, it is possible that the relatively modest inhibitory effect of CTL AKR103 treatment during the first few days after CTL injection was lost by the faster growth rate of the melAKR-Flu cells during the next days.

Other aspects that influence the success rate of adoptive transfer of CTL are the localization of the CTL to the tumor and T-cell survival in vivo. These variables determine the time frame in which the CTL must exert their cytolytic activity against the tumor cells. To investigate these aspects in our in vivo model, we analyzed the lungs of the treated and control mice at day 17 for the presence of injected CTL by flow cytometry. In our adoptive transfer experiments, a small population of injected viable CTLs that bound human CD45-specific and human CD8-specific antibodies, as well as specific tetramers, was still present in the lungs at day 17. The number of CTLs varied among mice within a group, but the average number did not differ significantly between groups of mice treated with the four different CTL clones (data not shown). This suggests that the greater tumor growth inhibition by the influenza virus-specific CTL clones, as compared with the MART-1-specific CTL clones, probably did not result from an increased tumor localization or T-cell survival. To follow the kinetics of CTL in the circulation and lungs after injection in more detail, we measured the presence of CTL clone INFA24 after 3 h, 3 days, 7 days, and 14 days after injection of $5 \times 10^6$ T cells in melAKR-Flu tumor-bearing mice (Fig. 5). Viable CTLs were detectable in the peripheral blood at a concentration of 25 cells/10,000 murine PBMC (0.25%), and $\sim 9,500$ injected T cells (0.18%) are present in the lungs during the first 3 days after injection, followed by rapid clearance between day 3 and day 7 after injection. The tumor cells in the lungs started to increase in number after day 3 when most of the injected CTLs were cleared. These results show that the injected INFA24 CTLs mediated the antitumor effect at very low E:T cell ratios and were able to effect up to 40% reduction in tumor growth (Fig. 5). Moreover, these results suggest that the CTLs mediated their antitumor effect during the first 3 days after injection.

We have described previously that ectopic hTERT expression immortalizes human T cells, and that the hTERT-transduced T cells remain dependent on TCR-mediated stimulatory signals to enter cell cycle in vitro (17, 18). Our in vivo results of the hTERT-transduced CTL detection in the lungs of the treated mice showed that the hTERT-transduced cells had not given rise to T-cell malignancies during the adoptive transfer experiments. These results suggest that hTERT-transduced CTLs had not started to grow in an uncontrolled manner upon injection in vivo. Because these experiments lasted only 17 days, we injected hTERT-transduced CTLs in a group of 14 nontumor-bearing mice and examined the presence of T cells after 3–11 months. Viable human CTLs were detectable in the lungs of these mice after 3 and after 6 months, albeit at low numbers. No viable T cells were found in the mice 9 or 11 months after CTL injection, which is probably due to the absence of exogenous rhIL-2 during this prolonged time of follow up. The mice did not have any abnormalities that might have been caused by the injection of hTERT-transduced T cells, suggesting that a part of the CTLs survived in these mice, but had not developed into malignancies within 11 months.

Tumor Growth Inhibition Mediated by hTERT-Transduced CTL, as Compared with Untransduced CTL. After having determined that the hTERT-transduced CTL clones are able to reduce the growth of tumors, it was important to determine whether the hTERT transduction reduced or potentiated the in vivo activities of the CTL clones. Therefore, we compared the effect of adoptive transfer of hTERT-transduced CTLs with untransduced CTLs. Due to the limited life span of cloned CTLs (15), sufficient cells of the untransduced CTLs for adoptive transfer could only be generated of clone INFA13. We performed this experiment in a novel model of noninvasive in vivo tumor growth detection in mice (33). This method would allow us to determine whether there would be differences between hTERT and untransduced CTLs in in vivo activities at different time points. To this end, melAKR-Flu cells were transduced with a retrovirus encoding the luciferase gene (melAKR-Flu-Luc), which allows detection of the tumor cells in vivo by the bioluminescence signal by the luciferase activity upon enzymatic conversion of the substrate luciferin. This method enables the monitoring of tumor growth during the experiment at multiple time points and represents a quantitative measure of the tumor load (33). Fig. 6 shows that the hTERT-transduced cells of clone INFA13 almost completely inhibited the growth of melAKR-Flu-Luc tumors in RAG/γcKO mice. This tumor growth inhibition was comparable with the effect of the untransduced CTLs of clone INFA13, indicating that hTERT transduction did not affect the in vivo efficacy of human CTLs in vivo. These results are consistent with our previously published in vitro data showing that ectopic hTERT ex-

![Image](cancerres.aacrjournals.org)
TUMOR GROWTH INHIBITION BY hTERT-TRANSDUCED T CELLS

Fig. 6. Bioluminescence imaging of tumor growth inhibition mediated by human telomerase reverse transcriptase-transduced or untransduced CTL. A, pseudocolor image representing the spatial distribution of photons counts within the animal (blue, least intense and red, most intense) overlayed on the gray-scale reference image, allowing anatomical localization of the melAKR-Flu-Luc tumors. Pictures show a representative picture of the tumor load at days 0, 3, 10, and 17 in untreated mice. B, RAG/γcKO mice injected with 0.5 × 10^6 melAKR-Flu-Luc tumor cells and treated after 3 days with a single dose of 5 × 10^5 hTERT-transduced INFA13 T cells (C), or untransduced INFA13 T cells (+), or left untreated (○). Tumor growth was monitored at days 4, 6, 10, 13, 17, and 20 by injection of luciferin and detection of the bioluminescence (BLU). Graph shows the average values of 5 mice/group. C, India ink staining of the lungs of melAKR-Flu-Luc tumor-bearing mice treated with untransduced INFA13 T cells (right); hTERT-transduced INFA13 T cells (middle); or untreated mice (left); bars, ±SD.

expression does not change the functional activity of human T cells (17, 18, 24).

**DISCUSSION**

In the present report we have shown that hTERT-transduced human CTLs are capable of inhibiting human tumor growth in vivo. A single dose of CTL was already sufficient to mediate significant reduction in tumor growth, and tumor growth inhibition was dependent on specific recognition of the tumor cells by the CTL. To our knowledge, this is the first report of the in vivo functional activity of hTERT-transduced human T cells. Our results with hTERT-transduced human CTL in vivo are consistent with the results of human CTL therapy in other xenograft models in immunodeficient mice, showing tumor growth inhibition by the infused CTL (1, 3–5). However, the mice used in these previous studies were nude mice, or SCID mice, in which minimal levels of T- and B-cell development or the presence of natural killer cells may have hampered interpretation of the antitumor effect mediated by the infused T cells. We used mice that completely lacked T, B, and natural killer cells. Moreover, RAG/γcKO mice do not develop thymomas, which usually occur in NOD/SCID mice, allowing the use of these mice in long-term experiments. Most reports on adoptive transfer of human CTLs in xenograft models describe the antitumor effect of one single CTL population or CTL clone in vivo (1, 3–5). In the present study, we have compared different human CTL clones for their growth-inhibiting effect in one tumor model, and observed that infusion of CTL clones, which are functionally active in vitro, can have variable outcomes in vivo. It is important to note here that these experiments could only be made because the hTERT transduction allowed for generation of unlimited numbers of CTLs.

Clinical studies have shown the success of adoptive transfer of human T cells to mediate tumor regression in melanoma and renal cell carcinoma patients. The potential application and success rate of adoptive transfer depends, however, on the isolation of tumor-reactive T cells of each patient. Moreover, successful treatment may require a more diverse population of both CD8+ and CD4+ T cells with various antigen specificities. Multiepitope targeting by T cells may avoid immune escape of tumor cells that have lost the expression of the targeted antigen. Furthermore, the addition of CD4+ T cells to the infused cells has been shown to enhance human CTL graft survival in immunodeficient mice (34), and may be important to maintain CTL effector function. To apply adoptive transfer of mixtures of well-defined monoclonal T-cell populations to more patients, it is important to expand a large portion of the patient-derived T-cell population to select for T cells recognizing the tumor cells. Ectopic hTERT expression allows the expansion of human T cells to enable long-term adoptive treatment with repeated doses of T cells without the limitation of the life span of human T cells. Therefore, T cells can be selected for the tumor reactivity upon hTERT transduction without additional selection on replicative age. We have described previously that hTERT-transduction does not change the antigen specificity and functional activity of human CD8+ and of CD4+ T cells in vitro. In the present report we show that hTERT-transduced CTL clones are capable of inhibiting tumor growth in vivo and mediate tumor growth inhibition to the same extent as the untransduced CTL. Therefore, ectopic hTERT expression allows the expansion of human CTL without affecting the in vivo functionality. hTERT-transduced T cells may be considered for application in adoptive transfer procedures, provided appropriate assessment of the possible risk of hTERT-transduced T cells to acquire a malignant phenotype in vivo. To decrease this risk and in view of the observation that hTERT expression is not required for in vivo function, additional research will focus on ways to eliminate the ectopic hTERT expression in the T cells after large-scale expansion and before adoptive transfer.

Our in vivo study has shown several aspects of adoptive transfer of human T cells that influence the in vivo efficacy. When comparing different CTL clones both in vitro and in vivo, we have observed that the in vitro assays, such as specific tetramer binding and cytotoxicity assays were not fully predictive of the in vivo efficacy of CTL clones (Table 1). Both influenza virus-specific CTL clones, INFA24 and INFA3, equally lysed melAKR-Flu cells in vitro, and displayed equal functional avidity, IFNγ production, and tetramer binding. However,
CTL clone INFA13 was significantly more effective in vivo than clone INFA24. This difference was not apparent from the above-mentioned in vitro assays and may be the result of other yet-to-be-defined functional differences of the CTL that may affect the in vivo activity. These findings illustrate the limitations of in vitro assays to predict in vivo efficacy of CTL and the importance of testing CTLs for their functionality in vivo. Furthermore, although melAKR-Flu tumor cells were equally well killed in vitro by both the influenza virus-specific CTL clones and the MART-1-specific CTL clones, the influenza virus-specific CTL clones produced more IFNγ upon recognition of melAKR-Flu cells. These results may indicate a rate-limiting effect in the cytotoxicity assays caused by the level of sensitivity of target cells to lysis by CTL, which, therefore, do not reveal quantitative differences between highly lytic CTL clones. The importance of the CTL avidity for the in vivo efficacy upon adoptive transfer was demonstrated for tyrosinase-related protein 2-specific murine CTL in a syngeneic setting than the parental CTL (35), suggesting a positive gene was found to be more effective in mediating tumor regression in vivo. The elevated level of IFNγ in vivo in the influenza virus-specific CTL in mediating tumor growth inhibition in vivo. The elevated level of IFNγ production by the influenza virus-specific CTL upon recognition of the melAKR-Flu tumor cells may have additionally enhanced the in vivo efficacy of these clones. Local IFNγ production in the tumor leads to an increased HLA class I expression on tumor cells, allowing better tumor cell recognition by the CTL. Adoptive transfer of murine CTL transduced with the IFNγ gene was found to be more effective in mediating tumor regression in a syngeneic setting than the parental CTL (35), suggesting a positive effect of IFNγ production by the CTL on the therapeutic efficiency.

In conclusion, the discrepancies found between in vitro assays and in vivo antitumor effect of CTL (Table 1) suggest that the efficacy of CTL clones to eradicate tumors in vivo depends on the balance between CTL effector function and tumor growth in vivo. The adoptive transfer therapy model described in the present report reveals the efficacy of human CTL clones in vivo, and allows selection of CTLs capable of mediating superior therapeutic effects. Furthermore, our model allows evaluating adoptive transfer strategies of CD8+ T cells combined with tumor-specific CD4+ T cells or dendritic cells to enhance the antitumor effect.

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