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 life 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 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 INF13 inhibited the growth of melAKR-Flu tumors in vivo and not of the parental melAKR melanoma cells. Furthermore, the hTERT-transduced CTL clone INFA13 inhibited tumor growth to the same extent in vivo as the untransduced CTL clone, 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 AKR4D8 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 vivo, 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 10⁷–10¹⁰ 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 10⁷–10⁹ 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 tumors of culture-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

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Note: N. Verra and A. Jorritsma contributed equally to this work. K. Weijer, A. Voordouw, and H. Spits are currently at the Department of Cell Biology and Histology, AMC, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. J. Ruizendaal and E. Hooijberg are currently at the Department of Pathology, VUMC, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands. R. Luiten is currently at the Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, E3-Q, Albinusdreef 2, P.O. Box 9600, 2300 RC Leiden, The Netherlands.
Requests for reprints: Hergen Spits, Department of Cell Biology and Histology, AMC, University of Amsterdam, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands. Phone: 31-20-5564966; Fax: 31-20-6974156; E-mail: hergen.spits@amc.uva.nl.
Materials and Methods

Mice. Male RAG-2⁻/⁻ IL-2Rγ⁻/⁻ (RAG/γKO) mice on a C57/Bl6 background, as described previously (25), were used at the age of 6–8 weeks. These mice have 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). MelAKR-Flu was generated by transduction of melAKR with the retrovirus encoding the influenza matrix peptide 58–66 (GILGFVFTL) that binds to HLA-A2 molecules, as described (17). Briefly, T cells were transduced with the feeder cell mixture containing pho-tohemagglutinin, 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 (Retronecstor, 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.

Chromium Release Assay. The cytotoxic activity of the CTL clones was tested in a 4-h ⁵¹Cr release assay with 500 targets/well and E:T ratios ranging from 1:1 to 30:1, as described (31). Unlabeled K562 cells were added in a 50-fold excess to suppress nonspecific target cell lysis by the T-cell clones.

1 E. Hooiberg and J. J. Ruizendaal, unpublished observations.

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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^{-5} to 10^{-8}. ELISAs were performed in a volume of 150 μl/test. β-Cr 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 (EAAGIGILTV) 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 IFN-γ by ELISA. The concentration of IL-4, IL-10, or IFN-γ in the supernatant was determined in triplicate cultures in a 96-well plate using a standard curve of diluted recombinant cytokine provided by the influenza virus matrix peptide 58–66 (GILGFVFTL) for clones INFA13 and INAF24, or with the MART-1 peptide 26–35 (EAAGIGILTV) 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 IFN-γ by ELISA. The concentration of IL-4, IL-10, or IFN-γ 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 Rag2γcKO 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. rhl-2 depot in the flank. This rhl-2 depot consists of a suspension of 2 × 10^6 IU rhl-2 in 40 μl Iscove’s modified Dulbecco’s medium (Invitrogen Life Technologies), and 80 μl incomplete Freund’s adjuvant (Difco Laboratories, Detroit, MI). 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 rhl-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 Dickenson), PerCP-conjugated antihuman CD8 Ab (Becton Dickenson), and allophycocyanin-conjugated antihuman CD45 Ab (Becton Dickenson) for 30 min on ice. Human T cells were detected as the population-expressing GFP and human CD45, but not murine CD45, which were also tested in parallel for human CD8 expression. The kinetics of the injected human T cells was measured in groups of 8 Rag2γcKO 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 the peripheral blood. In vivo bioluminescence. 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 rhl-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-antirabbit 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 visualization 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-YPF configuration. MelAKR-Flu tumor cells were transduced with a retrovirus encoding the luciferase-ires-YPF 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 T cells and 10^6 melAKR-Flu-Luc cells or hTERT-transduced cells of CTL clone INFA13, as well as a s.c. rhl-2 depot. Control mice received only tumor cells and the rhl-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 μg/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 overlayed on the gray-scale reference image, allowing anatomic 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 four of the CTL clones were transduced with the hTERT-IRES-YPF encoding retrovirus. During subsequent culture, we observed an accumulation of hTERT-IRES-GFP-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-IRES-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 × 10⁶ 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 cytolysis 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

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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 ⁵¹Cr release assay. Non-specific 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 cell 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 ⁵¹Cr 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 pM 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 ELA-GIGILTV, 100 nM -1 µM EAAGIGILTV. Clone AKR4D8: 1–10 pM 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 pM at an E:T ratio of 5:1. Graphs show an equal avidity of clone INFA13 and INFA24 at 100 pM.
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-TCRαβ 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 un-transduced 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/-yKO Mice.** We developed a transplantation protocol of human melanoma cells in the RAG/-yKO mice. When injected i.v. in the tail vein, melAKR melanoma cells were found to grow as microtumors in the lungs after 17 days (Fig. 3A). The microtumors were growing in between the alveoli, extravasating from the blood vessels (Fig. 3B), as well as in the pleural cavity. The number of lung microtumors varied between 200 and 900 in two entire longitudinal lung sections in different experiments. Injection of 5 × 10⁶ melAKR cells gave rise to tumors in all of the mice, which were sufficient 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 0.5 × 10⁶ melAKR-Flu cells per mouse gave rise to lung microtumors at day 17 that were equivalent in number to those obtained with 1 × 10⁶ melAKR cells. Therefore, experiments involving melAKR-Flu cells were performed with 0.5 × 10⁶ 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 × 10⁶ 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 microtumors 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.
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 AKR103 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

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

Fig. 3. Growth of human melanoma cells after i.v. injection in RAG/cKO mice. A, growth of melAKR cells in the lungs of untreated mice 17 days after injection of 1 × 10^6 cells. Original magnification, ×100. Injection of 0.5 × 10^6 melAKR-Flu cells gave similar results. B, detail of microtumors in the lungs showing diffuse tumor growth in the alveoli and the growth of microtumors in blood vessels. Original magnification, ×400. C, reduction of the number and size of melAKR-Flu microtumors in the lungs of mice treated with influenza virus-specific CTL clone INFA24, as compared with untreated controls. Original magnification, ×100.

Fig. 4. Reduction of tumor growth by adoptive transfer of specific CTL clones in vivo. A, tumor growth inhibition of melAKR-Flu tumors in groups of 8 RAG/cKO mice treated with a single (1) dose of CTL clone INFA24 ( ), or a single (1), or two (2) doses of clone INFA13 ( ). ■, absence of tumor growth inhibition in the treatment of melAKR tumors with one or three doses of CTL clone INFA24. The average number of lung micro-tumors (left graph) and the total tumor size (right graph) in the control group was set at 100%, and the number and size of the tumors in the treated mice were calculated relative to the untreated control group for each experiment. Graphs show the average relative tumor growth reduction of three independent experiments. bars, ±SD. Significant decreases, as tested by the student t test, are indicated by *: one dose of clone INFA24 versus untreated mice, number of tumors: P = 0.009, tumor size: P = 0.001; one dose or two doses of clone INFA13 versus untreated mice, number of tumors: P = 0.000 and P = 0.000, tumor size: P = 0.002 and P = 0.001, respectively. B, tumor growth inhibition of melAKR tumor cells in RAG/cKO mice treated with a single (1) dose or three (3) doses of CTL clone AKR4D8 ( ) or clone AKR103 ( ). □, treatment of melAKR-Flu tumor-bearing mice with one or three doses of clone AKR103. Graphs show the relative average number of microtumors in the lungs (left) or the relative total tumor size (right) at day 17 in three independent experiments with groups of 8 mice, as described in A. Significant decreases in tumor growth are indicated by *: three doses of clone AKR4D8 versus untreated mice, number of tumors: P = 0.048, tumor size: P = 0.029; one or three doses of clone AKR103 versus untreated mice, number of tumors: P = 0.005 and P = 0.000, tumor size: P = 0.039 and P = 0.008, respectively.
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 × 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 ~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 CTL 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-
pression 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/ycKO 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 vitro (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 transfer 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,

<table>
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<th>Table 1 Correlation between results of in vitro functional assays and in vivo antitumor efficacy</th>
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<td><strong>CTL clone</strong></td>
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<tr>
<td>Lysis of melAKR cells in vitro</td>
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<td>Inhibition of melAKR tumor growth in vivo</td>
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<td>Lysis of melAKR-Flu cells in vitro</td>
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<td>Inhibition of melAKR-Flu tumor growth in vivo</td>
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<tr>
<td>IFNγ production*</td>
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<td>Specific tetramer binding</td>
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*In cocultures with melAKR-Flu cells.
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 mouse model (7) and for gp100-specific human CTL clones demonstrated for tyrosinase-related protein 2-specific murine CTL in vivo. The influenza virus-specific CTL displayed a higher tetramer expression on tumor cells, allowing better tumor cell recognition by effector function and tumor growth in vivo. The adoptive transfer therapy demonstrated in the present report reveals the efficacy of human CTL clones for the treatment of melanoma metastases in SCID/hu mice. Cancer Res 1993;53:1038–44.


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